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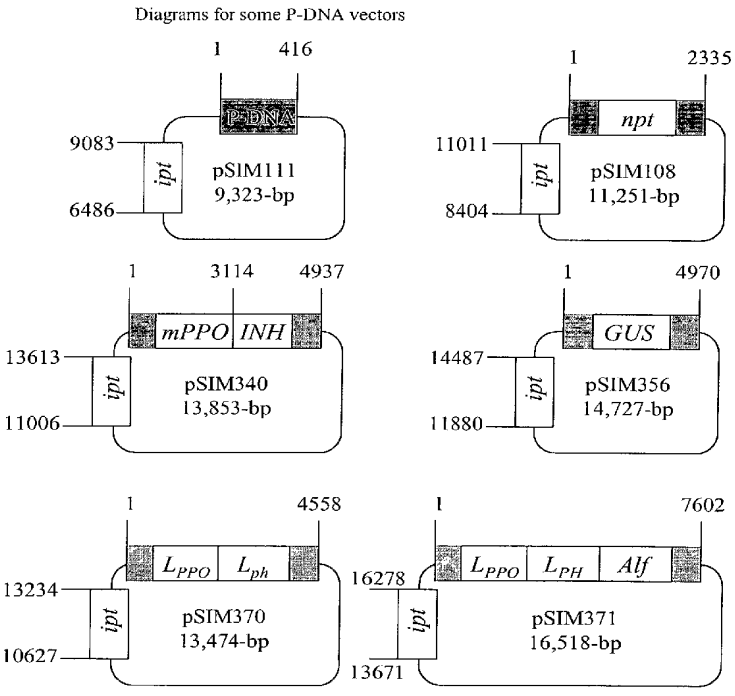

(57) Abstract: The present invention relates to a new plant breeding process. The process improves the agronomic performance of crop plants by using genetic material that is also used in classical breeding. Instead of sexually recombining entire genomes at random, as is done in classical breeding, specific genetic elements are rearranged *in vitro* and inserted back into individual plant cells. Plants obtained through this new plant breeding process do not contain foreign nucleic acid but only contain nucleic acid from the plant species selected for transformation or plants that are sexually compatible with the selected plant species. Plants developed through this new plant breeding process are provided. In particular, potato plants displaying improved tuber storage and health characteristics are provided.

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PRECISE BREEDING

FIELD OF THE INVENTION

This application claims priority to U.S. provisional application serial numbers 60/357,661 and 60/377,602, which are incorporated herein by reference. The present invention relates to methods for improving the nutritional, health, and agronomic characteristics of a plant by modifying specific, well-characterized DNA in the plant's genome. As opposed to classical plant breeding, the inventive process does not introduce unknown or potentially toxic genes into the plant genetic make-up. Furthermore, the inventive method, unlike conventional genetic engineering strategies, does not incorporate nucleic acids from foreign species, *i.e.*, species that are not inter-fertile with the plant to be modified by genetic engineering, into the plant genome. Plants developed through the inventive plant breeding process display improved agronomic characteristics. Particularly preferred plants of the present invention include potatoes that exhibit improved health and tuber storage characteristics, and turfgrasses that exhibit improved disease and drought tolerance.

BACKGROUND

The agronomic performance of plants has typically been improved by either classical plant breeding or genetic engineering. Classical breeding typically results in the transfer of unknown nucleic acids from one plant to another. Genetic engineering techniques introduce foreign nucleic acids into the plant genome, *i.e.*, DNA that is not from a plant or that is not from a plant that is naturally interfertile with the plant to be modified by genetic engineering. For example, genetic engineering introduces non-plant nucleic acids into a plant genome. Both classical breeding and genetic engineering strategies create plant genomes that contain undesirable and unwanted genetic material, and the resultant cross-bred or transgenic plants can exhibit unfavorable traits. The inadequacies of both strategies can prove harmful to the transgenic plants, as well as to the animals and humans who consume such products.

Conventional breeding relies on the transfer of unknown DNA

Plant breeding typically relies on the random recombination of plant chromosomes to create varieties that have new and improved characteristics. Thus, by screening large populations of progeny that result from plant crosses, breeders can identify those plants that display a desired trait, such as an increase in yield, improved vigor, enhanced resistance to diseases and insects, or greater ability to survive under drought conditions. However, classical breeding methods are laborious and time-consuming, and new varieties typically display only relatively modest improvements.

Furthermore, classical plant breeding typically results in the transfer of hundreds of unknown genes into a plant genome. It is likely that some of those transferred genes encode potentially harmful allergens, such as patatin, lectins, chitinases, proteases, thaumatin-like proteins, lipid transfer proteins, amylases, trypsin inhibitors, and seed storage proteins (Breiteneder et al., *J Allergy Clin Immunol* 106: 27-36).

Similarly, introgressed genes can be involved in the biosynthesis of toxins including lathyrogens, hydrazines, glucosinolates and goitrogens, cumarins, saponins, alkaloids, glycoalkaloids, biogenic amines, enzyme inhibitors, such as lectins (haemagglutinins), trypsin inhibitors, chelating substances such as phytates and oxalates, ribotoxins, antimicrobial peptides, amino acids such as beta-N-oxalylamino-L-alanine, atractyloside, oleandrine, taxol, and isoquinoline (Pokorny, *Cas Lek Cesk* 136: 267-70, 1997). The risk of inadvertently introducing such poisons into human and animal food supplies is further increased through efforts to "untap" the genetic diversity of wild crop relatives that have not been used before for food consumption (Hoisington et al., *Proc Natl Acad Sci U S A* 96: 5937-43, 1999).

Although classical plant breeding can easily introduce genes involved in undesirable anti-nutritional compounds into food crops and plants, it cannot easily remove them. For instance, it took about 15 years to reduce harmful phytate levels in corn and rice by inactivating *Lpa* genes (Raboy, *J Nutr* 132: 503S-505S, 2002). The long timeframe for realizing positive results is not practical, especially since there is an urgent need for methods that more effectively and efficiently improve the quality of

food crops. One example of a gene that only recently was found to be associated with the synthesis of anti-nutritional compounds is the polyphenol oxidase (*PPO*) gene, which oxidizes certain phenolic compounds to produce mutagenic, carcinogenic and cytotoxic agents like phenoxyl radicals and quinoid derivatives (Kagan et al., *Biochemistry* 33: 9651-60, 1994). The presence of multiple copies of this gene in the genome of plants such as potato makes it particularly difficult to reduce PPO activity through breeding.

Even more time is needed for the removal of anti-nutritional compounds if little or nothing is known about their genetic basis. For instance, no genes have been linked to the accumulation of high concentrations of acrylamide, a potent neurotoxin and mutagen, in some potatoes that are heated to 160^0C or higher (Tareke et al., *J Agric Food Chem.* 50: 4998-5006, 2002). It is therefore very difficult to efficiently develop new potato varieties that produce less acrylamide during processing using conventional breeding. Thus, there is a need to grow potatoes and other carbohydrate-rich foods, such as wheat, with reduced levels of such dangerous compounds, but without the use of unknown or foreign nucleic acids.

Other anti-nutritional compounds that can accumulate during processing and are difficult to minimize or eliminate through breeding include the Maillard-reaction products N-Nitroso-N-(3-keto-1,2-butanediol)-3'-nitrotyramine (Wang et al., *Arch Toxicol* 70: 10-5, 1995), and 5-hydroxymethyl-2-furfural (Janzowski et al., *Food Chem Toxicol* 38: 801-9, 2000). Additional Maillard reaction products that have not been well characterized are also known to display mutagenic properties (Shibamoto, *Prog Clin Biol Res* 304: 359-76, 1989).

It can be equally difficult to rapidly increase levels of positive nutritional compounds in food crops due to the inherent imprecision of conventional plant breeding. For instance, it would be desirable to increase levels of "resistant starch" (Topping et al., *Physiol Rev* 81: 1031-64, 2001) in a variety of crops. Such starch is ultimately responsible for promoting immune responses, suppressing potential pathogens, and reducing the incidence of diseases including colorectal cancer (Bird et al., *Curr Issues Intest Microbiol* 1: 25-37, 2000). However, the only available plants with increased

levels of resistant starch are low-yielding varieties like maize mutants "amylose extender", "dull", and "sugary-2." Creation of new high resistant starch sources, such as potato, would enable broader dietary incorporation of this health-promoting component.

The inability to safely manipulate the genotypes of plants often leads to the use of external chemicals to induce a desired phenotype. Despite numerous breeding programs to delay tuber sprouting, for example, no potato varieties are available commercially that can be stored for months without treatment with sprout inhibitors. The latter, such as isopropyl-N-chlorophenyl-carbamate (CIPC), is linked to acute toxicity and tumor development, and can be present in processed potato foods at concentrations between 1 mg/kg and 5 mg/kg.

Genetic engineering relies on the transfer of foreign DNA

Genetic engineering can be used to modify, produce, or remove certain traits from plants. While there has been limited progress in improving the nutritional value and health characteristics of plants, most improvements target plant traits that promote ease of crop cultivation. Thus, certain plants are resistant to the glyphosate herbicide because they contain the bacterial gene 5-enolpyruvylshikimate-3-phosphate synthase (Padgette et al., *Arch Biochem Biophys.* 258: 564-73, 1987). Similarly, genetic engineering has produced insect-, viral-, and fungal-resistant plant varieties (Shah et al., *Trends in Biotechnology* 13: 362-368, 1995; Gao et al., *Nat Biotechnol.* 18: 1307-10, 2000; Osusky et al., *Nat Biotechnol.* 18: 1162-6, 2000), but few with enhanced nutrition or health benefits.

According to standard, well-known techniques, genetic "expression cassettes," comprising genes and regulatory elements, are inserted within the borders of *Agrobacterium*-isolated transfer DNAs ("T-DNAs") and integrated into plant genomes. Thus, *Agrobacterium*-mediated transfer of T-DNA material typically comprises the following standard procedures: (1) *in vitro* recombination of genetic elements, at least one of which is of foreign origin, to produce an expression cassette for selection of transformation, (2) insertion of this expression cassette, often together

with at least one other expression cassette containing foreign DNA, into a T-DNA region of a binary vector, which usually consists of several hundreds of basepairs of *Agrobacterium* DNA flanked by T-DNA border sequences, (3) transfer of the sequences located between the T-DNA borders, often accompanied with some or all of the additional binary vector sequences from *Agrobacterium* to the plant cell, and (4) selection of stably transformed plant cells. *See, e.g.*, U.S. Patent Nos. 4,658,082, 6,051,757, 6,258,999, 5,453,367, 5,767,368, 6,403,865, 5,629,183, 5,464,763, 6,201,169, 5,990,387, 4,693,976, 5,886,244, 5,221,623, 5,736,369, 4,940,838, 6,153,812, 6,100,447, 6,140,553, 6,051,757, 5,731,179, 5,149,645 and EP 0 120,516, EP 0 257,472, EP 0 561,082, 1,009,842A1, 0 853,675A1, 0 486,233B1, 0 554,273A1, 0 270,822A1, 0 174,166A1, and WO 01/25459.

Thus, genetic engineering methods rely on the introduction of foreign nucleic acids into the food supply. Those techniques transfer complex fusions of a few to more than 20 genetic elements isolated from viruses, bacteria, and plants, that are not indigenous to the transformed plant species. Such foreign elements include regulatory elements such as promoters and terminators, and genes that are involved in the expression of a new trait or function as markers to identify or select for transformation events. Despite the testing of foods containing foreign DNA for safety prior to regulatory approval, many consumers are concerned about the long-term effects of eating foods that express foreign proteins, which are produced by genes obtained from other, non-plant species.

One commonly used regulatory element is the 35S "super" promoter of cauliflower mosaic virus (CaMV), which is typically used in plant engineering to induce high levels of expression of transgenes to which it is directly linked. However, the 35S promoter also can enhance the expression of native genes in its vicinity (Weigel et al., *Plant Physiol.*, 122: 1003-13, 2000). Such promoters may thus induce unpredictable alterations in the expression of endogenous genes, possibly resulting in undesirable effects such as increased alkaloid production. Preferred "strong" promoters are generally those isolated from viruses, such as rice tungro bacilliform virus, maize streak virus, cassava vein virus, mirabilis virus, peanut chlorotic streak

caulimovirus, figwort mosaic virus and chlorella virus. Other frequently used promoters are cloned from bacterial species and include the promoters of the nopaline synthase and octopine synthase gene.

To obtain appropriate termination of gene translation, terminator sequences are fused to the 3'-end of transgenes and include genetic elements from the nopaline synthase and octopine synthase genes from *Agrobacterium*. Other genetic elements may be used to further enhance gene expression or target the expressed protein to certain cell compartments. These elements include introns to boost transgene expression and signal peptide sequences to target the foreign gene to certain cellular compartments, often derived from foreign plant species.

Certain genes involved in expression of a new trait are most frequently derived from foreign sources. If native genes are used, they are often inverted to silence the expression of that gene in transgenic plants and co-transformed with foreign DNA such as a selectable marker. The main disadvantage of this "antisense" technology is that the inverted DNA usually contains new and uncharacterized open reading frames inserted between a promoter and terminator. Thus, potato plants that were genetically modified with antisense constructs derived from the starch related gene *R1* (Kossmann et al., *US Patent* 6,207,880), the L- and H-type glucan phosphorylase genes (Kawchuk et al., *US Patent* 5,998,701, 1999), the polyphenol oxidase gene (Steffens, *US Patent* 6,160,204, 2000), and genes for starch branching enzymes I and II (Schwall et al., *Nature Biotechnology* 18: 551-554, 2000) all potentially express new peptides consisting of at least 50 amino acids (Table 1). These new peptides may interfere with plant development and/or reduce the nutritional value of potato, and are therefore undesirable.

Conventional marker genes are incorporated into genetic constructs and used to select for transformation events. They confer either antibiotic or herbicide resistance (U.S. Patent No. 6,174,724), a metabolic advantage (U.S. Patent No. 5,767,378), or a morphologically abnormal phenotype (U.S. Patent No. 5,965,791) to the transformed plant. Such markers are typically derived from bacterial sources.

Furthermore, because of the infidelity of T-DNA transfer, about 75% of transformation events in plants such as tomato, tobacco, and potato contain plasmid "backbone" sequences in addition to the T-DNA (Kononov et al., *Plant J.* 11: 945-57, 1997). The presence of such backbone sequences is undesirable because they are foreign and typically contain origins of replication and antibiotic resistance gene markers.

There do exist various methods for removing elements like foreign marker genes, but few are easily applicable to plant genetic engineering. According to one such method, the marker gene and desired gene or nucleotide sequence are placed on different vectors. The infection of plants with either a single *Agrobacterium* strain carrying both vectors (U.S. Patent No. 6,265,638) or two *Agrobacterium* strains each of which carries one of the vectors can occasionally result in unlinked integration events, which may be separated genetically through outbreeding. The main disadvantage of this method is that the genetic separation of loci can be very laborious and time-consuming, especially if T-DNA integration events are linked. Furthermore, this method is not widely applicable in apomictic plants, which reproduce asexually, such as Kentucky bluegrass, or vegetatively propagated crops such as potato, which cannot be readily bred due to inbreeding depression, high levels of heterozygosity, and low fertility levels.

Another method for removing foreign genetic elements relies on inserting the foreign gene, like the selectable marker, into a transposable element. The modified transposable element may then be spliced out from the genome at low frequencies. Traditional crosses with untransformed plants must then be performed to separate the transposed element from the host (U.S. Patent No. 5,482,852). As described for the previous method, this alternative method cannot be used for vegetatively propagated or apomictic plant systems.

A third method of removing a marker gene uses the Cre/lox site-specific recombination system of bacteriophage P1 (Dale & Ow, *Proc. Natl. Acad. Sci. USA*, 88: 10558-62, 1991). Insertion of a marker gene together with the *Cre* recombinase gene and a chimeric gene involved in induction of Cre (both with their own promoters

and terminators) between two lox sites leads to excision of the region delineated by the lox sites during the regeneration process (Zuo et al., *Nat. Biotechnol.*, 19: 157-61, 2001). This complicated process is inefficient and not reliable, and may cause genome instability.

Recent studies report that some plant genes themselves may be used as transformation markers. Examples of such plant markers include Pga22 (Zuo et al., *Curr Opin Biotechnol.* 13: 173-80, 2002), Cki1 (Kakimoto, *Science* 274: 982-985, 1996) and Esr1 (Banno et al., *Plant Cell* 13: 2609-18, 2001). All of the genes, however, trigger cytokinin responses, which confer an undesirable phenotype to the transformed plant. Furthermore, such plant markers would still need to be removed upon transformation by any of the methods described above.

Alternative methods to transform plants are also based on the *in vitro* recombination of foreign genetic elements, and rely on bacterial plasmid sequences for maintenance in *E. coli*, parts of which are co-integrated during the transformation process. Examples of such methods to transform plants with foreign DNA are described in U.S. Patent Nos. 5,591,616, 6,051,757, 4,945,050, 6,143,949, 4,743,548, 5,302,523, and 5,284,253.

Marker-free transgenic plants may also be obtained by omitting any selection procedures prior to regeneration. A disadvantage of this method is that most events generated through this method will represent untransformed or chimeric plants because they will usually not be derived from single transformed plant cells. It is extremely difficult and laborious to use a marker-free procedure for the identification of transgenic plants that contain the same DNA insertion(s) in all their cells.

Thus, there is a very important need to improve plants beyond that which can be accomplished through the classical breeding crosses and conventional genetic engineering techniques, and which does not rely on the insertion of unknown or foreign nucleic acid into a plant genome. Accordingly, the present invention provides methods and compositions for precisely modifying a plant's own genetic material. Thus, the inventive "precise breeding" strategy does not induce undesirable

phenotypes and does not introduce unknown or foreign nucleic acid into a plant genome.

SUMMARY

The present invention provides methods of genetically enhancing the nutritional value and agronomic performance of a plant without the permanent or stable incorporation of either unknown or foreign DNA into the genome of that plant. According to the methods of the present invention, specific, well-characterized nucleic acids, gene elements, and genes are isolated from a desired plant species or from a plant species that is sexually compatible with the desired plant, modified, and then reinserted back into the genome of the desired plant species. The modification may entail mutating the isolated nucleic acid sequence, deleting parts of the isolated nucleic acid, or simply joining the isolated nucleic acid to another polynucleotide, such as subcloning the isolated nucleic acid into a plasmid vector.

Accordingly, transgenic plants produced by the inventive methodology do not possess genomes that comprise any foreign species' nucleic acids. Thus, the methods of the present invention produces a transgenic plant whose genome does not comprise a non-plant species promoter, does not comprise a non-plant species terminator, does not comprise a non-plant species 5'-untranslated region, does not comprise a non-plant species 3'-untranslated region, does not comprise a non-plant species marker gene, does not comprise a non-plant species regulatory element, does not comprise a non-plant species gene, and does not comprise any other polynucleotide that is obtained from a non-plant species genome.

Thus, the present invention provides a method for producing a stable transgenic plant that exhibits a modified phenotype that is not exhibited by the non-transformed plant, comprising (a) transforming plant cells with a desired polynucleotide; (b) growing plants from the transformed cells; and (c) selecting a plant stably transformed with said desired polynucleotide which exhibits a new phenotype that is not exhibited by plants grown from the corresponding non-transformed plant cells. Preferably, the desired polynucleotide consists essentially of (i) nucleic acid sequences that are

isolated from and/or native to the genome of the plant cells, or to other plants of the same species, or are isolated from and/or native to the genome of a plant species that is sexually compatible with the plant from which the plant cells were isolated; and (ii) at least one DNA sequence that is a border-like sequence that has a sequence that is native to the genome of said plant cells or is native to the genome of plant cells of the same species, or is native to a plant that is sexually compatible with the plant from which the plant cells were isolated, and wherein the border-like sequence is capable of stably integrating the desired polynucleotide into the genome of said plant cells.

A preferred method of the present invention entails producing a transgenic plant that exhibits a modified phenotype that is not exhibited by the non-transformed plant, comprising (a) infecting explants with *Agrobacterium* carrying (i) a "P-DNA" vector, which contains a desired polynucleotide that is native to the transgenic plant, and (ii) a "LifeSupport" vector that contains an expression cassette containing a selectable marker gene; (b) selecting for transient expression of the selectable marker gene, preferably for 1-10 days, for 3-7 days, or for 4-5 days; (c) transferring explants to regeneration media to allow shoot formation; (d) screening populations of shoots to determine which comprise at least one copy of the desired polynucleotide in their genomes and, of those, which shoots do not contain any foreign nucleic acids, such as the selectable marker gene, in their genomes; and (e) allowing shoots which contain the desired polynucleotide in their genomes but not any marker gene DNA, to develop into whole plants, wherein the resultant whole plants exhibit a modified phenotype that is not exhibited by plants grown from non-transformed plant cells of the same species.

According to such a method, the desired polynucleotide (i) consists essentially of only elements that are isolated from and/or native to the genome of the plant cell species or sexually compatible species thereof; (ii) comprises at least one border element that has a sequence that is isolated from, or native to, the genome of the plant cell species or sexually compatible species thereof, and is capable of stably integrating the desired polynucleotide into the genome of a plant cell exposed to the vector; and (iii) is stably

integrated into the genome of the transformed plant; wherein the method does not integrate non-plant species or foreign DNA into the genome of the transformed plant.

Furthermore, any selectable marker gene may be used as an indicator of successful transformation. For instance, a "neomycin phosphotransferase" marker gene, or an "hpt" marker gene may be used to confer resistance to the aminoglycoside antibiotics, kanamycin and hygromycin respectively. Other marker genes include the "bar" marker gene, which confers resistance to herbicide phosphinothricin; the "DHFR" marker gene, which confers resistance to methotrexate; and the "ESPS" marker gene, which confers resistance to Round-up herbicide. It is well known in the art how to follow expression of such marker genes to determine whether or not the marker gene has been stably expressed into the genome of a transformed plant cell. Accordingly, the skilled artisan knows how to follow expression of the marker gene to determine that the marker gene is only transiently expressed in the transformed plant cell.

In another aspect of the invention, there is provided a method of making a stably transformed plant comprising the steps of: (1) identifying a target gene; (2) isolating a leader or trailer DNA sequence associated with said target gene; (3) optionally modifying said isolated leader or trailer DNA; (4) operably linking said leader or trailer DNA to native regulatory elements to form an expression cassette; (5) inserting said expression cassette into a P-DNA that is located on a binary vector, wherein the binary vector also carries an operable cytokinin gene such that the inadvertent insertion of additional binary vector sequences, which are of foreign origin, are detected by expression of the cytokinin gene; (6) introducing the modified binary vector into *Agrobacterium*; (7) stably integrating the rearranged native DNA into the genomes of plant cells using LifeSupport-mediated transformation; (8) regenerating plant cells that contain the rearranged native DNA; (9) discarding plants that display a cytokinin-overproducing phenotype and do not fully regenerate; and (10) maintaining for further analysis the desirable plants that are indistinguishable from untransformed plants.

In another aspect of the instant invention, a method of modifying the expression of a trait in a selected plant species is provided. In one embodiment, the method

comprises (1) identifying the trait to be modified; (2) constructing a recombinant DNA molecule consisting essentially of genetic elements isolated from, or native to, the selected plant species, wherein the recombinant DNA molecule, when integrated into the genome of the selected plant species, modifies the expression of the trait in the transformed plant species; (3) stably integrating the recombinant DNA molecule into cells of the selected plant species using LifeSupport-mediated transformation; and (4) identifying transformed plants exhibiting modified expression of the trait.

In a preferred embodiment, polynucleotide that is native to a desired plant is inserted into the desired plant's genome by infecting explants with two different *Agrobacterium* strains. A first *Agrobacterium* strain is capable of transferring the native DNA from P-DNA vectors to plant cells; a second strain can transfer a T-DNA carrying an expression cassette for a selectable marker gene to plant cells. Examples of the latter vector include the so-called, "LifeSupport" vectors described herein. By preferably selecting plants that transiently express the marker gene for 1-10 days, for 3-7 days, or for 4-5 days, and subsequently transferring explants to regeneration media, a population of events is obtained, part of which represents plants that contain at least one copy of the polynucleotide, but which lack any copies of the T-DNA or marker gene.

In another embodiment, a single *Agrobacterium* strain is used that carries both a P-DNA vector, which houses the desired, native gene of interest or polynucleotide between P-DNA border-like sequences, and a LifeSupport vector, which contains a marker gene. The marker gene may, or may not, be inserted between P-DNA border-like sequences, T-DNA border sequences, or other T-DNA-like border sequences.

Thus, in another preferred embodiment, the P-DNA vector contains at least two expression cassettes, one of which comprises a native screenable or selectable marker gene driven by a native promoter and followed by a native terminator.

By preferably selecting for at least 2 days and more preferably for at least 5 days for native marker gene expression and subsequently transferring explants to regeneration media, a population of events is obtained that represent plants containing at least one

copy of the introduced DNA stably integrated into their genomes. In preferred embodiments, the plant-derived marker gene encodes a mutant 5-enolpyruvul-3-phosphoshikimic acid synthase or tryptophan decarboxylase. In a more preferred embodiment, the selectable marker encodes for salt tolerance. In a most preferred embodiment, the salt tolerance gene has the nucleotide sequence shown in SEQ ID 35 and is used to select for transformation events in potato.

In yet another embodiment, the modified expression of the trait is characterized by an increase in expression, a decrease in expression, or in undetectable expression.

In another aspect of the instant invention, a plant made by the method of (1) identifying the trait to be modified; (2) constructing a recombinant DNA molecule consisting essentially of genetic elements isolated from the selected plant species, wherein the recombinant DNA molecule when integrated into the genome of the selected plant species modifies the expression of the trait in the transformed plant species; (3) stably integrating the recombinant DNA molecule into cells of the selected plant species through LifeSupport-mediated transformation; and (4) identifying transformed plants exhibiting modified expression of the trait, is provided.

In a further aspect, a method of modifying expression of a trait in a selected plant species is provided. This method comprises (1) identifying the trait to be modified; (2) constructing a recombinant DNA molecule consisting essentially of (a) genetic elements isolated from the selected plant species, wherein the genetic elements when integrated into the genome of the selected plant species modifies the expression of the trait in the transformed plant species; and (b) a selectable marker gene that is isolated from the same plant species; (3) stably integrating the recombinant DNA molecule into cells of the selected plant species through LifeSupport-mediated transformation; (4) detecting the selectable marker gene; and (5) identifying transformed plants exhibiting modified expression of the trait.

In yet one other aspect, a plant exhibiting a modified expression of a trait is provided. In one embodiment, the plant has stably integrated into its genome a recombinant DNA molecule consisting essentially of genetic elements isolated from a plant of the

same species, or from a plant that is sexually compatible with that species, wherein the recombinant DNA molecule modifies the expression of the trait.

In another aspect of the present invention, an isolated nucleotide sequence referred to as "plant-DNA" ("P-DNA") is provided. In a preferred embodiment, the P-DNA itself lacks any genes or parts thereof and is delineated by terminal, T-DNA "border-like" sequences that share at least 50%, at least 75%, at least 90% or at least 95% sequence identity with the nucleotide sequence of the T-DNA borders of any virulent *Agrobacterium* strain, and which support an efficient transfer of the entire P-DNA from *Agrobacterium* to plant cells.

In a preferred embodiment a "border-like" sequence promotes and facilitates the integration of a polynucleotide to which it is linked. In another preferred embodiment, each terminal sequence of the modified P-DNA is between 5-100 bp in length, 10-80 bp in length, 15-75 bp in length, 15-60 bp in length, 15-50 bp in length, 15-40 bp in length, 15-30 bp in length, 16-30 bp in length, 20-30 bp in length, 21-30 bp in length, 22-30 bp in length, 23-30 bp in length, 24-30 bp in length, 25-30 bp in length, or 26-30 bp in length. More preferably, the border-like sequence is between 20 and 28 nucleotides in length.

In a preferred embodiment, the P-DNA left and right border sequences of the present invention are isolated from and/or are native to the genome of a plant that is to be modified and are not identical in nucleotide sequence to any known *Agrobacterium*-derived T-DNA border sequence.. Thus, in one embodiment, a P-DNA border sequence may possess 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more nucleotides that are different from a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Alternatively, in another embodiment, a P-DNA border, or a border-like sequence of the present invention has at least 95%, at least 90%, at least 80%, at least 75%, at least 70%, at least 60% or at least 50% sequence identity with a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. More preferably, a native plant P-DNA border sequence that shares greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%,

92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% nucleotide sequence identity with an *Agrobacterium* T-DNA border sequence.

In another preferred embodiment, a border-like sequence can be isolated from a plant genome and then modified or mutated to change the efficiency by which they are capable of integrating a nucleotide sequence into another nucleotide sequence. In another embodiment, other polynucleotide sequences may be added to or incorporated within a border-like sequence of the present invention. Thus, in yet another embodiment, a P-DNA left border or a P-DNA right border may be modified so as to possess 5'- and 3'- multiple cloning sites, or additional restriction sites. In a further embodiment, a P-DNA border sequence may be modified to increase the likelihood that backbone DNA from the accompanying vector is not integrated into the plant genome.

In an even more preferred embodiment, the P-DNAs are isolated from any plant by using degenerate primers in a polymerase chain reaction. In one preferred embodiment, the P-DNA is derived from potato, is delineated by 25-bp termini with 80 and 88% identity to conventional T-DNA borders, respectively, and has the nucleotide sequence shown in SEQ ID NO. 1. In another most preferred embodiment, the P-DNA is derived from wheat, is delineated by 25-bp termini with 72% and 92% identity with conventional T-DNA borders, respectively, and contains the nucleotide sequence shown in SEQ ID NO. 34.

Such a P-DNA may be modified so as to comprise other polynucleotides positioned between the border-like sequences. In a preferred embodiment, the modified P-DNA consists essentially of, in the 5'- to 3'- direction, a first border-like sequence that promotes DNA transfer, a promoter, a desired polynucleotide that is operably linked to the promoter, a terminator and a second border-like sequence that also promotes DNA transfer. In one other embodiment, the desired polynucleotide represents one or several copies of a leader, a trailer or a gene in sense and/or antisense orientations. In

a more preferred embodiment, the modified P-DNA contains expression cassettes for both a mutant *PPO* gene and an invertase inhibitor gene.

Thus, in a preferred embodiment, the desired polynucleotide comprises a sense and antisense sequence of a leader sequence. In a more preferred embodiment, the leader sequence is associated with a gene that is endogenous to a cell of the selected plant species. In yet a more preferred embodiment, the leader is associated with a gene that is selected from the group consisting of a *PPO* gene, an *R1* gene, a type L or H alpha glucan phosphorylase gene, an UDP glucose glucosyltransferase gene, a HOS1 gene, a S-adenosylhomocysteine hydrolase gene, a class II cinnamate 4-hydroxylase gene, a cinnamoyl-coenzyme A reductase gene, a cinnamoyl alcohol dehydrogenase gene, a caffeoyl coenzyme A O-methyltransferase gene, an actin depolymerizing factor gene, a *Nin88* gene, a *Lol p 5* gene, an allergen gene, a P450 hydroxylase gene, an ADP-glucose pyrophosphorylase gene, a proline dehydrogenase gene, an endo- 1,4-beta-glucanase gene, a zeaxanthin epoxidase gene, and a 1-aminocyclopropane-1-carboxylate synthase gene.

In yet another preferred embodiment, the desired polynucleotide sequence comprises a sense and antisense sequence of a trailer sequence. In a preferred embodiment, the trailer sequence is associated with a gene selected from the group consisting of a *PPO* gene, an *R1* gene, a type L or H alpha glucan phosphorylase gene, an UDP glucose glucosyltransferase gene, a *HOS1* gene, a S-adenosylhomocysteine hydrolase gene, a class II cinnamate 4-hydroxylase gene, a cinnamoyl-coenzyme A reductase gene, a cinnamoyl alcohol dehydrogenase gene, a caffeoyl coenzyme A O-methyltransferase gene, an actin depolymerizing factor gene, a Nin88 gene, a Lol p 5 gene, an allergen gene, a P450 hydroxylase gene, an ADP-glucose pyrophosphorylase gene, a proline dehydrogenase gene, an endo- 1,4-beta-glucanase gene, a zeaxanthin epoxidase gene, and a 1-aminocyclopropane-1-carboxylate synthase gene.

In a preferred embodiment, the desired polynucleotide, such as a gene, is isolated from, and/or is native to the plant that is to be transformed. In another preferred embodiment, the desired polynucleotide is modified or mutated. In one embodiment, a mutation to the isolated polynucleotide may render the desired nucleotide greater than

or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% dissimilar to its unmutated form.

In a preferred embodiment of the present invention, the promoter of an expression cassette located within a P-DNA is a constitutive promoter. In a more preferred embodiment the constitutive promoter is the promoter of the Ubiquitin-3 gene of potato. In an even more preferred embodiment the constitutive promoter is the promoter of the Ubiquitin-7 gene of potato.

In another embodiment, the promoter of an expression cassette located within a P-DNA is a regulatable promoter. In a more preferred embodiment, the regulatable promoter is sensitive to temperature. In an even more preferred embodiment, the regulatable promoter is a ci21A promoter or a C17 promoter, each isolated from potato (Schneider et al., *Plant Physiol.* 113: 335-45, 1997; Kirch et al., *Plant Mol Biol* 33: 897-909, 1997).

In another embodiment, the promoter of an expression cassette located within a P-DNA can be regulated in a temporal fashion. In a preferred embodiment, the promoter is an rbcS promoter (Ueda et al., *Plant Cell* 1: 217-27, 1989).

In yet another embodiment, the promoter of an expression cassette located within a P-DNA is regulated by any one of abscisic acid, wounding, methyl jasmonate or gibberellic acid. In a further embodiment, this promoter is a promoter selected from either a Rab 16A gene promoter, an α-amylase gene promoter or a pin2 gene promoter.

In another embodiment, the promoter of an expression cassette located within a P-DNA is a tissue-specific promoter. In a particularly preferred embodiment, this promoter is a GBSS promoter isolated from *S. tuberosum*.

In one embodiment, the present invention provides a P-DNA vector that is capable of replication in both *E.coli* and *Agrobacterium*, and contains either a P-DNA or a

modified P-DNA. In a preferred embodiment, this vector also contains an expression cassette for a cytokinin gene in its backbone to enable the selection against backbone integration events.

In another preferred embodiment, the desired nucleotide sequence further comprises a spacer element. In a more preferred embodiment, the spacer element is a Ubi intron sequence or a GBSS spacer sequence.

In another preferred embodiment, the desired nucleotide sequence comprises a mutated native gene encoding a functionally inactive protein, which reduces the overall activity of that protein if expressed in transgenic plants. In yet a more preferred embodiment, this mutated gene encodes a functionally inactive polyphenol oxidase lacking a copper binding domain.

In another preferred embodiment, the desired nucleotide sequence comprises a native gene encoding a functionally active protein. In yet a more preferred embodiment, this gene encodes for a protein with homology to the tobacco vacuolar invertase inhibitor.

In another embodiment, the terminator of an expression cassette located within a P-DNA is a Ubi3 terminator sequence or a 3'-untranslated region of a gene of a selected plant species.

In another aspect of the instant invention, a method for modifying a target plant cell is provided. In one embodiment, the method comprises: (1) inserting a modified P-DNA into the genome of at least one cell in the target plant cell using LifeSupport-mediated transformation; and (2) observing if there is a phenotypic change in the target plant cell; wherein the promoter in the modified P-DNA transcribes the sense and/or antisense untranslated sequences associated with a native gene to reduce expression of that native gene, thereby modifying the target plant cell. In another preferred embodiment, the promoter in the modified P-DNA transcribes a gene to overexpress that gene in the target plant cell.

In yet another aspect, there is provided a method of making a transgenic plant cell of a selected plant species that contains a modified P-DNA. The method comprises co-

transfecting a plant cell of the selected plant species with a P-DNA vector and a LifeSupport vector that comprises a marker gene flanked by a T-DNA left border and a T-DNA right border and a mutant *virD2* gene inserted into the vector backbone, and selecting for a plant cell that transiently expresses the marker gene, and isolating a plant cell that contains the modified P-DNA integrated into its genome but does not contain any nucleotides from the LifeSupport vector. In a preferred embodiment, the marker gene confers resistance to kanamycin. In a most preferred embodiment the yeast ADH terminator follows the kanamycin resistance gene.

In a preferred embodiment, the plant cell of the selected plant species targeted for transformation is in culture. In another preferred embodiment, the plant cell of the selected plant species targeted for transformation is within a plant.

The present invention also provides a plant of the selected species that comprises at least one cell with a genome that contains a modified P-DNA. In a preferred embodiment, the modified P-DNA consists essentially of, in the 5'- to 3'- direction, a first terminus that functions like a T-DNA border followed by P-DNA sequences, a promoter, a desired nucleotide sequence operably linked to both a promoter, a terminator and additional P-DNA sequences delineated by a second terminus. In another embodiment, the desired polynucleotide represents one or several copies of a leader, a trailer and a gene in the sense and/or antisense orientation.

In another embodiment, a plant that comprises at least one cell with a genome that contains a modified P-DNA is envisioned.

In another aspect of the invention, a method for reducing the expression of a gene in a selected plant species is provided. The method comprises the LifeSupport-mediated transformation of a plant cell from a selected plant species with a P-DNA vector, wherein the modified P-DNA of this vector is stably integrated into the genome of the plant cell. In another aspect of the invention, the modified P-DNA comprises a desired polynucleotide that reduces expression of an endogenous gene from the selected plant species.

In another aspect of the instant invention, a gene native to the selected plant species may be mutated and reintroduced into the plant using the inventive methods. Preferably, the mutated gene, for instance a mutated *PPO* gene, is integrated into the plant cell genome using a P-DNA vector.

The present invention also provides a method for reducing the undesirable expression of the polyphenol oxidase gene in a selected plant species. In a preferred embodiment, the method comprises integrating into a genome of a selected plant species a modified P-DNA comprised only of nucleotide sequences isolated from the selected plant species or from a plant that is sexually compatible with the selected plant species, consisting essentially of, in the 5'- to 3'- direction, a first P-DNA terminus that functions like a T-DNA border followed by flanking P-DNA sequences; a promoter; a desired nucleotide which is a sense-oriented trailer nucleotide sequence associated with a specific *PPO* gene; an antisense-oriented sequence of the trailer nucleotide sequence from the specific *PPO* gene; a termination sequence, and additional P-DNA sequences delineated by a second terminus that functions like a T-DNA border, wherein the promoter produces a double-stranded RNA molecule that reduces the expression of the specific *PPO* gene, thereby reducing black spot bruising in specific tissues of the plant. In another embodiment, the sense- and antisense-oriented nucleotide sequences from the leader nucleotide sequences are obtained from the 5'-untranslated region preceding the specific *PPO* gene. In a further embodiment, the sense- and antisense-oriented leader or trailer sequence associated with the *PPO* gene may be separated by another polynucleotide sequence, referred to herein, as either an intron or a "spacer." In a preferred embodiment, the leader or trailer sequence is associated with a potato *PPO* gene. In a more preferred embodiment, the leader or trailer sequence is associated with a potato *PPO* gene that is expressed in potato tubers. In a most preferred embodiment, the leader or trailer sequence is associated with a potato *PPO* gene that is expressed in all parts of the potato tuber except for the epidermis.

The present invention also provides a method for reducing acrylamide production, sprout-induction during storage, phosphate accumulation, and/or cold-induced sweetening in tubers of a selected plant species.

In a preferred embodiment, the method comprises the LifeSupport-mediated transformation of a selected plant species with a modified P-DNA comprised only of nucleotide sequences isolated from the selected plant species, or from plants that are sexually compatible with the selected plant species, consisting essentially of, in the 5'- to 3'- direction, a first P-DNA with a left border-like sequence, a promoter, a desired nucleotide sequence, which is a sense-oriented nucleotide sequence from the leader sequence associated with the *R1* gene, an antisense-oriented sequence from this leader sequence, a termination sequence, and a right border-like sequence. Upon expression, a leader-RNA duplex is produced that reduces expression of the *R1* gene, thereby reducing cold-induced sweetening in the plant. In another embodiment, the desired sense- and antisense-oriented nucleotide sequences represent the trailer associated with the *R1* gene. In a further embodiment, the sense- and antisense-oriented leader or trailer associated with *R1* may be separated by another polynucleotide sequence, referred to herein, as either an intron or a "spacer."

In another preferred embodiment, the method comprises the LifeSupport-mediated transformation of a selected plant species with a modified P-DNA that is similar to the one described above but contains a leader- or trailer sequence associated with an alpha glucan phosphorylase gene.

In yet another preferred embodiment, the method comprises the LifeSupport-mediated transformation of a selected plant species with a modified P-DNA that contains an invertase inhibitor gene.

In another preferred embodiment, the modified P-DNA described in the preceding paragraphs are used to reduce the accumulation of additional undesirable products of the Maillard reaction, which occurs during the heating of carbohydrate-rich foods such as potato tubers. These undesirable products include advanced glycation end products (AGEs) that have been associated with various pathologies.

The present invention also provides a method for increasing resistant starch levels in the storage organs of plants and food crops.

In a preferred embodiment, the method comprises the LifeSupport-mediated transformation of a selected plant species with a modified P-DNA that contains an expression cassette for a fusion of the trailer sequences associated with the starch branching enzyme I and II genes.

The present invention also provides isolated nucleotide sequences comprising the promoters of the potato GBSS gene and the potato proteinase inhibitor gene, which are predominantly expressed in tubers. The isolated promoters have the nucleotide sequence shown in SEQ ID NO.: 6 and SEQ ID NO.:40, respectively.

In one aspect, the present invention provides a method of modifying a trait of a selected plant comprising:

a. stably transforming cells from the selected plant with a desired polynucleotide, wherein the desired polynucleotide consists essentially of a nucleic acid sequence that is native to the selected plant, native to a plant from the same species, or is native to a plant that is sexually interfertile with the selected plant,

b. obtaining a stably transformed plant from the transformed plant cells wherein the transformed plant contains the desired polynucleotide stably integrated into the genome and wherein the desired polynucleotide modifies the trait.

In a preferred embodiment, the method further comprises co-transfecting the plant cells with a selectable marker gene that is transiently expressed in the plant cells, and identifying transformed plant cells, and transformed plants obtained from the transformed plant cells, wherein the selectable marker gene is not stably integrated and the desired polynucleotide is stably integrated into the genome.

In a preferred embodiment, the desired polynucleotide comprises a P-DNA, GBSS promoter, Ubi7 promoter, Ubi3 promoter, PIP promoter, modified PPO gene, invertase inhibitor gene, salt tolerance gene, R1-associated leader, phosphorylase-associated leader, R1-associated trailer, SBE-associated trailers, Ubi-intron, GBSS spacer, UbiT.

In another preferred embodiment, a "plant" of the present invention is a monocotyledenous plant, selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

In yet another embodiment, a "plant" of the present invention is a dicotyledenous plant, selected from the group consisting of avacado, potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

In yet another embodiment, plants and plant cells of the present inventive methods are transformed via *Agrobacterium*-mediated transformation. Preferably, the *Agrobacterium*-mediated transformation relies on the use of at least one binary vector. In yet another embodiment, the *Agrobacterium*-mediated transformation method uses a first binary vector and a second binary vector. In a preferred embodiment the first binary vector contains the desired polynucleotide and the second binary vector contains a selectable marker gene, wherein the selectable marker gene is operably linked to a promoter and a terminator.

According to the present methods, the trait that is modified is selected from the group consisting of enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased drought tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced cold and frost tolerance, enhanced color, enhanced sweetness, improved vigor, improved taste, improved texture, decreased phosphate

content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity.

The present invention also encompasses a plant made by the present methods.

In another aspect, the present invention provides a method of modifying a trait in a selected plant comprising:

(a) identifying the trait to be modified;

(b) constructing a first polynucleotide consisting essentially of native genetic elements isolated from the selected plant, a plant from the same species, or a plant that is sexually interfertile with the selected plant, wherein the native genetic elements are capable of modifying the expression of a gene that controls the trait

(c) constructing a second polynucleotide comprising a selectable marker gene that is operably linked to a promoter and a terminator;

(d) co-transfecting plant cells from the selected plant with the first and second polynucleotides;

(e) selecting for the transient expression of the selectable marker gene;

(f) screening for plant cells stably transformed with the first polynucleotide but do not contain the second DNA molecule integrated into the genome; and

(g) obtaining a stably transformed plant from the transformed plant cells that exhibit a modified expression of the trait.

In one embodiment, the genetic elements comprise at least one of a promoter, sequence of interest, terminator, enhancer, intron, spacer, or regulatory elements. In another embodiment, method of claim 4, wherein the plant cells are transfected with the first polynucleotide before the second polynucleotide or vice versa.

In one embodiment, the sequence of interest is a gene. In another embodiment, the gene is a mutated or wild-type polyphenol oxidase gene or a mutated or wild-type R1 gene. In one other embodiment, the sequence of interest is a leader or trailer sequence, wherein the leader or trailer sequence represents a sequence upstream or downstream of a gene that is native to the plant cell. In yet another embodiment, the sequence of interest comprises a sense-oriented leader sequence operably linked to an antisense leader sequence. In another embodiment, the sequence of interest comprises a sense-oriented trailer sequence operably linked to an antisense trailer sequence. In another embodiment, the promoter is an inducible promoter. In another embodiment, the terminator is a yeast ADH terminator sequence.

According to the present invention, a leader construct comprises in 5'-to 3'- direction, a promoter, a sense-oriented leader sequence, the antisense sequence of the leader, and a terminator, wherein expression of the leader construct produces a double-stranded RNA molecule that facilitates the down-regulation of expression of the gene to which it is associated. In one other embodiment, the leader sequence is associated with, and located upstream of, the coding region of the PPO gene, the R1 gene, an L-type phosphorylase gene, or an alpha glucan phosphorylase gene.

In another embodiment, the trailer construct comprises in 5'-to 3'- direction, a promoter, a sense-oriented trailer sequence, the antisense sequence of the trailer, and a terminator, wherein expression of the trailer construct produces a double-stranded RNA molecule that facilitates the down-regulation of expression of the gene to which it is associated. In a preferred embodiment, the trailer sequence is associated with, and located downstream of, the coding region of the PPO gene, the R1 gene, an L-type phosphorylase gene, or an alpha glucan phosphorylase gene.

The method further comprises exposing the plant cell to a second vector that comprises a marker element, wherein the marker is transiently expressed in the transformed plant and is not stably integrated into the genome of the transformed plant. In one embodiment, the marker is a herbicide resistance gene, an antibiotic resistance gene, or NPTII.

Preferably, the plant cells are transformed via *Agrobacterium*-mediated transformation. In one embodiment, the *Agrobacterium*-mediated transformation relies on the use of at least one binary vector. In yet another embodiment, the *Agrobacterium*-mediated transformation method uses a first binary vector and a second binary vector. In one other embodiment, the first binary vector carries the first polynucleotide and the second binary vector carries the second polynucleotide.

The present invention provides another method of modifying the expression of a gene in a selected plant comprising:

(a) identifying the functional gene;

(b) constructing a first polynucleotide consisting essentially of native genetic elements isolated from the selected plant, a plant of the same species as the selected plant, or a plant that is sexually interfertile with the selected plant, wherein the native genetic elements are capable of modifying the expression of the gene;

(c) constructing a second polynucleotide comprising a functional selectable marker gene;

(d) co-transfecting plant cells from the selected plant with the first and second poylnucleotides;

(e) selecting for the transient expression of the selectable marker gene;

(f) screening for plant cells stably transformed with the first polynucleotide but do not contain the second polynucleotide integrated into the genome; and

(g) obtaining a transformed plant from the transformed plant cells that exhibit modified expression of the gene.

Preferably, the plant cells are transformed via *Agrobacterium*-mediated transformation. In one embodiment, the *Agrobacterium*-mediated transformation relies on the use of at least one binary vector. In yet another embodiment, the

Agrobacterium-mediated transformation method uses a first binary vector and a second binary vector. In one other embodiment, the first binary vector carries the first polynucleotide and the second binary vector carries the second polynucleotide.

In another embodiment, the first polynucleotide comprises at least one of a P-DNA, GBSS promoter, Ubi7 promoter, Ubi3 promoter, PIP promoter, modified PPO gene, invertase inhibitor gene, salt tolerance gene, R1-associated leader, phosphorylase-associated leader, R1-associated trailer, SBE-associated trailers, Ubi-intron, GBSS spacer, UbiT.

In another embodiment, the second polynucleotide comprises at least one of a selectable marker gene, an omega-mutated virD2 polynucleotide, a codA polynucleotide, and a codA::upp fusion polynucleotide.

The present invention also encompases a plant made by such method.

In one other embodiment, a transgenic plant is provided which exhibits a modified expression of a trait compared to the non-trasgenic plant from which it was derived, wherein the transgenic plant is stably transformed with a desired polynucleotide consisting essentially of native genetic elements isolated from the plant, a plant in the same species, or a plant that is sexually interfertile with the plant, and wherein the polynucleotide modifies the expression of the trait.

In another preferred embodiment, the "plant" of the present invention is a monocotyledenous plant, selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

In yet another embodiment, the "plant" of the present invention is a dicotyledenous plant, selected from the group consisting of avacado, potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

In another embodiment, the trait is selected from the group consisting of enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased drought tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced cold and frost tolerance, enhanced color, enhanced sweetness, improved vigor, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity.

In another embodiment, the desired polynucleotide comprises at least one of a P-DNA, GBSS promoter, Ubi7 promoter, Ubi3 promoter, PIP promoter, modified PPO gene, invertase inhibitor gene, salt tolerance gene, R1-associated leader, phosphorylase-associated leader, R1-associated trailer, SBE-associated trailers, Ubi-intron, GBSS spacer, UbiT.

The present invention also encompasses an isolated, border-like nucleotide sequence ranging in size from 20 to 100 bp that shares between 52% and 96% sequence identity with a T-DNA border sequence from *Agrobacterium tumafaciens*. In a preferred embodiment, the isolated nucleotide sequence is isolated from a monocotyledenous plant, selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm. In another embodiment, the nucleotide sequence is isolated from a dicotyledenous plant selected from the group consisting of potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

In yet another embodiment, the isolated nucleotide sequence is isolated from potato, and has a nucleotide sequence shown in either SEQ ID NO. 94 or 95. In a preferred embodiment, the isolated nucleotide sequence shares 52% sequence identity with a T-DNA border sequence from *Agrobacterium tumafaciens*. The present invention encompasses a vector that comprises such nucleotide sequences.

The present invention also provides method of making a plant stably transformed with a desired polynucleotide comprising:

(a) isolating a P-DNA that is flanked by border-like sequences from the plant wherein the border-like sequences share between 52% and 96% sequence identity with an *Agrobacterium tumafaciens* T-DNA border sequence;

(b) inserting the desired polynucleotide between the P-DNA border-like sequences to form a P-DNA construct ; and

(c) transforming aa plant cell from the plant with the P-DNA construct; and

(d) recovering a plant from the transformed plant cell stably transformed with the P-DNA construct.

In one embodiment, the P-DNA construct is carried on a vector comprised of a backbone integration marker gene and transformed plant cells are selected that do not contain the backone integration marker gene. In another embodiment, the backbone integration marker gene is a cytokinin gene. In another embodiment, plant shoots are not selected that exhibit a cytokinin-overproducing phenotype. In yet another embodiment, the backnone integraton marker gene is the IPT gene, and plant shoots are not selected that exhibit an abnormal phenotype or cannot develop roots.

In one other embodiment, the plant cells are from a monocotyledenous plant selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

In another embodiment, the plant cells are from a dicotyledenous plant selected from the group consisting of potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

Preferably, the plant cells are transformed via *Agrobacterium*-mediated transformation. In one embodiment, the *Agrobacterium*-mediated transformation

relies on the use of at least one binary vector. In yet another embodiment, the *Agrobacterium*-mediated transformation method uses a first binary vector and a second binary vector. In one other embodiment, the first binary vector carries the first polynucleotide and the second binary vector carries the second polynucleotide. In one further embodiment, the second binary vector comprises at least one of a negative selectable marker gene and an omega-mutated virD2 gene, wherein the negative selectable marker gene is positioned within the right T-DNA border and the left T-DNA border, and wherein the omega-mutated virD2 gene is positioned within the backbone of the second binary vector. In a preferred embodiment, the second binary vector comprises both a negative selectable marker gene positioned within the right T-DNA border and the left T-DNA border, and an omega-mutated virD2 gene positioned within the backbone of the second binary vector.

The present invention also provides a P-DNA consisting essentially of, in the 5'- to 3'- direction, a first T-DNA border-like sequence, a promoter, a desired polynucleotide sequence operably linked to the promoter, a terminator, and a second T-DNA border-like sequence, wherein the border-like sequences have less than 100% sequence identity with T-DNA border sequences

In a preferred embodiment, the T-DNA border-like sequences, the promoter, the desired polynucleotide, and the terminator, are all isolated from the same plant, the same plant species, or plants that are sexually interfertile.

In another embodiment, the P-DNA further consists essentially of a selectable marker gene.

In yet another embodiment, the T-DNA border-like sequences, the promoter, the desired polynucleotide, the terminator and the selectable marker gene, are all isolated from the same plant, the same plant species, or plants that are sexually interfertile.

In yet another embodiment, the desired polynucleotide sequence in the P-DNA is a sequence upstream or downstream of the coding region of a gene, wherein the upstream sequence is a leader sequence, and wherein the downstream sequence is a trailer sequence. In this embodiment, the T-DNA border-like sequences, the

promoter, the leader sequence, the trailer sequence, the terminator and the selectable marker gene are all isolated from the same plant, the same plant species, or plants that are sexually interfertile.

In another embodiment, vectors comprising such P-DNA constructs are provided by the present invention.

In another embodiment, the promoter is a regulatable promoter. In yet another embodiment, the regulatable promoter is sensitive to temperature. In a preferred embodiment, the regulatable promoter is a wheat wcs120 promoter. In another embodiment, the promoter is under temporal regulation. In yet another embodiment, the promoter is a carboxylase promoter. In a further embodiment, the carboxylase promoter is a maize carboxylase promoter.

The promoter may be regulated by any one of abscisic acid, wounding, methyl jasmonate or gibberellic acid. In another embodiment, the promoter is a promoter selected from either a Rab 16A gene promoter, an α-amylase gene promoter or a pin2 gene promoter. In yet another embodiment, the promoter is a tissue-specific promoter.

In one other embodiment, the leader sequence is a part of a 5'- untranslated region of a gene that is endogenous to a cell of the selected plant species. In another embodiment, the 5'- untranslated region is upstream of a start codon of a gene that is selected from the group consisting of a PPO gene, an R1 gene, a HOS1 gene, a S-adenosylhomocysteine hydrolase gene, a class II cinnamate 4-hydroxylase gene, a cinnamoyl-coenzyme A reductase gene, a cinnamoyl alcohol dehydrogenase gene, a caffeoyl coenzyme A O-methyltransferase gene, an actin depolymerizing factor gene, a Nin88 gene, a Lol p 5 gene, an allergen gene, a P450 hydroxylase gene, an ADP-glucose pyrophosphorylase gene, a proline dehydrogenase gene, an endo- 1,4-beta-glucanase gene, a zeaxanthin epoxidase gene, and a 1-aminocyclopropane-1-carboxylate synthase gene.

In another embodiment, the trailer sequence is a part of the 3'-untranslated region of a gene that is downstream of a termination codon of a gene selected from the group consisting of a PPO gene, an R1 gene, a HOS1 gene, a S-adenosylhomocysteine hydrolase gene, a class II cinnamate 4-hydroxylase gene, a cinnamoyl-coenzyme A reductase gene, a cinnamoyl alcohol dehydrogenase gene, a caffeoyl coenzyme A O-methyltransferase gene, an actin depolymerizing factor gene, a Nin88 gene, a Lol p 5 gene, an allergen gene, a P450 hydroxylase gene, an ADP-glucose pyrophosphorylase gene, a proline dehydrogenase gene, an endo- 1,4-beta-glucanase gene, a zeaxanthin epoxidase gene, and a 1-aminocyclopropane-1-carboxylate synthase gene.

The present vector may further comprise a spacer element that is either an Ubi intron sequence or a GBSS spacer sequence. In another embodiment, the vector comprises a terminator that is a Ubi3 terminator sequence or a 3'-untranslated region of an endogenous plant gene.

In another embodiment, the vector comprises a selectable marker gene operably linked to a constitutive promoter and a Cre gene operably linked to an inducible promoter, wherein the selectable marker gene and the Cre gene are flanked by a first recombinase recognition site and a second recombinase recognition site. In another embodiment, the first recombinase recognition site and the second recombinase recognition site are lox sites.

In another embodiment, the inducible promoter is a temperature-sensitive promoter, a chemically-induced promoter, or a temporal promoter. In yet another embodiment, the inducible promoter is a Ha hsp17.7 G4 promoter, a wheat wcs120 promoter, a Rab 16A gene promoter, an α-amylase gene promoter, a pin2 gene promoter, a carboxylase promoter. In yet another preferred embodiment, further comprises a plant-derived marker gene. In another preferred embodiment, the plant-derived marker gene is an enolpyruvul-3-phosphoshikimic acid synthase gene.

In another aspect of the present invention, a method for modifying a plant cell is provided, comprising integrating a P-DNA sequence into the genome of a plant cell, wherein the P-DNA consists essentially of, in the 5'- to 3'- direction, a first T-DNA

border-like sequence, a promoter, a desired polynucleotide sequence operably linked to the promoter, a terminator, and a second T-DNA border-like sequence, wherein the border-like sequences have less than 100% sequence identity with T-DNA border sequences, and wherein the T-DNA border-like sequences, the promoter, the desired polynucleotide, and terminator, are all isolated from or native to the genome of the plant cell, wherein the desired polynucleotide comprises sense and antisense seqeunces of a leader sequence or trailer sequence that are associated with the upstream or downstream non-coding regions of a gene in the plant, and wherein expression of the desired polynucleotide produces a double-stranded RNA transcript that targets the gene associated with the desired polynucleotide, thereby modifying the plant cell.

The present invention also encompasses a method for modifying a plant, comprising:

(i) transfecting at least one cell in the plant with the vector of the present invention;

(ii) selecting a cell expressing the functional selectable marker;

(iii) isolating the cell expressing the functional selectable marker;

(iii) inducing the expression of the functional Cre gene in the isolated cell;

(iv) culturing the isolated cell; and

(ii) observing the phenotype of cultured cells;

wherein a phenotype that is different to an untransfected plant cell indicates that the target plant cell has been modified.

In a preferred embodiment the selecting step of this and other methods of the present invention is performed by identifying which cells are resistant to an antibiotic.

In another aspect, a method for identifying a target plant cell whose genome contains a P-DNA, comprises co-transfecting a plant target cell with the vector of the present invention and a second *Agrobacterium*-derived vector that comprises a marker gene flanked by a T-DNA left border and a T-DNA right border and a omega-mutated

virD2 gene, wherein the P-DNA is integrated into the genome of the plant target cell, and wherein no part of the second *Agrobacterium*-derived vector is integrated into the genome of the plant target cell. In a preferred embodiment, the marker in the second *Agrobacterium*-derived vector is a neomycin phosphotransferase gene.

In another aspect, the method for identifying a target plant cell whose genome contains at least a part of an integration cassette is provided, further comprises selecting cells that survive temporary growth on a kanamycin-containing media, wherein the genomes of the selected cells contain only the integration cassette. In one embodiment, the target plant cell is within a plant. A plant comprising at least one cell whose genome comprises such a P-DNA is also encompassed by the present invention.

The present invention also encompasses a plant comprising at least one cell whose genome is artificially manipulated to contain only plant-derived nucleic acids, wherein no cells of the plant contain foreign nucleic acids integrated into the cell genome.

The present invention also encompasses a polynucleotide comprising the polynucleotide sequence of SEQ ID NO. 93, wherein the polynucleotide is between 20 and 80 nucleotides in length. In one embodiment, the polynucleotide is between 21 and 70 nucleotides in length, between 22 and 50 nucleotides in length, between 23 and 40 nucleotides in length, or between 24 and 30 nucleotides in length.

In another aspect, the invention encompasses a tuber-specific promoter as shown in SEQ ID NO. 40.

The present invention also encompasses an *Agrobacterium*-based method of making transgenic plant cells that do not contain a selectable marker gene stably integrated in nuclear DNA comprising:

a. constructing a first binary vector comprised of a polynucleotide consisting essentially of a desired functional gene operably linked to T-DNA borders or T-DNA border-like sequences at the 5' and 3' ends of the desired functional gene;

b. constructing a second binary vector comprised of a functional selectable marker gene operably linked to T-DNA borders or T-DNA border-like sequences at the 5' and 3' ends of the functional selectable marker gene;

c. incubating plants cells with:

i. an *Agrobacterium* strain carrying the first and the second binary vectors; or

ii. a first *Agrobacterium* strain carrying the first binary vector and a second *Agrobacterium* strain carrying the second binary vector;

d. selecting plant cells wherein the desired functional gene is integrated into plant nuclear DNA without integration of the selectable marker gene into plant nuclear DNA following incubation for an appropriate time period on a medium containing an appropriate selection agent.

In a preferred embodiment, the selectable marker gene is a herbicide resistance gene or an antibiotic resistance gene. In another preferred embodiment, the antibiotic resistance gene is the nNPTII gene. In another embodiment, the antibiotic resistance gene is the npt II structural gene operably linked to the promoter from the Ubiquitin-7 gene and the termninator from yeast alcohol dehydrogenase 1 (ADH1) gene. According to this method, the plant cells are first incubated with the first *Agrobacterium* strain and then subsequently incubated with the second *Agrobacterium* strain or vice versa.

In a preferred embodiment, the first binary vector further comprises a binary integration marker gene that can be used to detect plant cells stably transformed with binary vector backbone sequences. In another embodiment, the binary vector

integration marker gene is selected from the group consisting of herbicide resistance gene, antibiotic resistance gene, or NPTII. In yet another embodiment, the second binary vector further comprises a gene fusion between the bacterial cytosine deaminase (*codA*) and uracil phophoribsyltransferase (*upp*) genes, which is inserted between the T-DNA or T-DNA border-like sequences, and plant cells are exposed to 5-fluorocytosine following incubation with the first and second *Agrobacterium* strains in order to select against those plant cells transformed with the second binary vector.

In yet another embodiment, the secondary binary vector further comprises a gene that reduces the probability of backbone integration. In one embodiment, such a gene is the omega-mutated *virD2* gene, wherein the omega-mutated *virD2* gene reduces the frequency of integration of the selectable marker gene into the plant nuclear DNA.

The present invention also encompasses an isolated nucleotide sequence comprising the GBSS promoter isolated from *S. tuberosum*. In a preferred embodiment, this isolated nucleotide sequence has the nucleotide sequence that is SEQ ID. NO. 6 or 13.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic illustrations of some P-DNA vectors used in the present invention. P-DNA region is indicated as grey box. "*ipt*" = expression cassette for the *ipt* gene; "*npt*" = expression cassette for the *nptII* gene; "*mPPO*" = expression cassette for a modified *PPO* gene; "*INH*" = expression cassette for an invertase inhibitor gene; "*GUS*" = expression cassette for the *GUS* gene; "*LPPO*" = expression cassette for a sense and antisense copy of the leader associated with a *PPO* gene; "*LPH*" = expression cassette for a sense and antisense copy of the leader associated with a phosphorylase gene; "*Alf*" = expression cassette for a potato *Alfin* homolog. See text for details.**Figure 2.** Gene-free expression cassettes

Figure 3. Alignment of potato and tobacco invertase inhibitor proteins. "St" = *Solanum tuberosum* (potato); "Nt" = *Nicotiana tabacum* (tobacco)

Figure 4. Alignment of trailers associated with various *PPO* genes.

Figure 5. Schematic illustrations of some LifeSupport vectors used in the present invention. "codA" is an expression cassette for the *codA* gene; "*codA::upp*" is an expression cassette for the *codA* gene fused to *upp*; "ΩvirD2" is an expression cassette for the *ΩvirD2* gene.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The "precise breeding" strategy of the present invention improves the agronomic performance, nutritional value, and health characteristics of plants and crops without introducing unknown nucleic acid, or nucleic acid from a foreign species into a plant species genome, and without producing undesirable phenotypes or harmful side-effects.

Thus, the present invention provides a transgenic plant, and methods for making such a plant that do not integrate nucleic acid from non-plant species into that plant's genome. Nucleic acids, promoters, regulatory elements, other non-coding gene sequences, markers, polynucleotides, and genes that are integrated into the selected plant genome are all preferably isolated from the plant that is to be transformed, plants of the same species to be transformed, or plants that are sexually interfertile with the plant to be transformed. Such "native" nucleic acids can be mutated, modified or cojoined with other native nucleic acids in an expression cassette and reintegrated into the selected plant genome, according to the methods described herein. Accordingly, the genotype and phenotype of the transgenic plant is altered using only that selected plant's own nucleic acid, or using nucleic acid from a plant that is sexually compatible with the selected plant.

To facilitate the production of such transgenic plants, the present invention makes use of the fact that not all T-DNA vectors used in *Agrobacterium*-mediated transformation are actually integrated into the plant genome; *i.e.*, while a vector may be taken up by the plant cell, an actual integration event may not occur. According to the present invention, one may use such a vector to carry a selectable marker gene into a plant cell. Plant cells can then be screened to determine whether the marker has been stably integrated into the plant genome by determining for how long the marker

gene is expressed. Accordingly, plant cells that only transiently express the selectable marker gene are desired because they represent cells that took up, but did not integrate into their genomes, the selectable marker gene.

Thus, by co-transforming a plant with such a "marker vector" and also with another vector that contains the desired native gene or polynucleotide, one can select plant cells that took up both vectors and, from those, determine which cells possess genomes that contain only the desired gene or polynucleotide. The "marker vector" can be modified to further reduce the possibility that the marker will be integrated into the plant genome. The present invention provides such "marker vectors" in the form of "LifeSupport" vectors.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein, and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described herein, are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, microbial culture, cell culture, tissue culture, transformation, transfection, transduction, analytical chemistry, organic synthetic chemistry, chemical syntheses, chemical analysis, and pharmaceutical formulation and delivery. Generally, enzymatic reactions and purification and/or isolation steps are performed according to the manufacturers' specifications. The techniques and procedures are generally performed according to conventional methodology disclosed, for example, in *Molecular cloning a laboratory manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), and *Current protocols in molecular biology*, John Wiley & Sons, Baltimore, MD (1989).

Amino acid sequence: as used herein, includes an oligopeptide, peptide, polypeptide, or protein and fragments thereof, that are isolated from, native to, or naturally occurring in a plant, or are synthetically made but comprise the nucleic acid sequence of the endogenous counterpart.

Artificially manipulated: as used herein, "artificially manipulated" means to move, arrange, operate or control by the hands or by mechanical means or recombinant means, such as by genetic engineering techniques, a plant or plant cell, so as to produce a plant or plant cell that has a different biological, biochemical, morphological, or physiological phenotype and/or genotype in comparison to unmanipulated, naturally-occurring counterpart.

Asexual propagation: producing progeny by generating an entire plant from leaf cuttings, stem cuttings, root cuttings, tuber eyes, stolons, single plant cells protoplasts, callus and the like, that does not involve fusion of gametes.

Backbone: nucleic acid sequence of a binary vector that excludes the T-DNA or P-DNA sequence intended for transfer.

Border and Border-like sequences: "border sequences" are specific *Agrobacterium*-derived sequences. Typically, a left border sequence and a right border sequence flank a T-DNA and they both function as recognition sites for *virD2*-catalyzed nicking reactions. Such activity releases nucleic acid that is positioned between such borders. See Table 2 below for examples of border sequences. The released nucleic acid, complexed with virD2 and virE2, is targeted to plant cell nuclei where the nucleic acid is often integrated into the genome of the plant cell. Usually, two border sequences, a left-border and a right-border, are used to integrate a nucleotide sequence that is located between them into another nucleotide sequence. It is also possible to use only one border, or more than two borders, to accomplish integration of a desired nucleic acid in such fashion.

According to the present invention, a "border-like" sequence is isolated from the selected plant species that is to be modified, or from a plant that is sexually-compatible with the plant species to be modified, and functions like the border sequences of *Agrobacterium*. That is, a border-like sequence of the present invention promotes and facilitates the integration of a polynucleotide to which it is linked. A plant-DNA, *i.e.*, P-DNA, of the present invention preferably contains border-like sequences.

A border-like sequence of a P-DNA is between 5-100 bp in length, 10-80 bp in length, 15-75 bp in length, 15-60 bp in length, 15-50 bp in length, 15-40 bp in length, 15-30 bp in length, 16-30 bp in length, 20-30 bp in length, 21-30 bp in length, 22-30 bp in length, 23-30 bp in length, 24-30 bp in length, 25-30 bp in length, or 26-30 bp in length.

The border-like sequences of the present invention can be isolated from any plant, such as from potato and wheat. See SEQ ID NO. 1 and SEQ ID NO. 34, for sequences which contain, at either end, the border-like sequences isolated from potato and wheat respectively. Thus, a P-DNA left and right border sequences of use for the present invention are isolated from and/or native to the genome of a plant that is to be modified. A P-DNA border-like sequence is not identical in nucleotide sequence to any known *Agrobacterium*-derived T-DNA border sequence.. Thus, a P-DNA border-like sequence may possess 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more nucleotides that are different from a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. That is, a P-DNA border, or a border-like sequence of the present invention has at least 95%, at least 90%, at least 80%, at least 75%, at least 70%, at least 60% or at least 50% sequence identity with a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, but not 100% sequence identity. As used herein, the descriptive terms "P-DNA border" and "P-DNA border-like" are exchangeable.

A native P-DNA border sequence is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, 60%, 59%, 58%, 57%, 56%, 55%, 54%, 53%, 52%, 51% or 50% similar in nucleotide sequence to a *Agrobacterium* a T-DNA border sequence. A border-like sequence can, therefore, be isolated from a plant genome and be modified or mutated to change the efficiency by which they are capable of integrating a nucleotide sequence into another nucleotide sequence. Other polynucleotide sequences may be added to or incorporated within a border-like

sequence of the present invention. Thus, a P-DNA left border or a P-DNA right border may be modified so as to possess 5'- and 3'- multiple cloning sites, or additional restriction sites. A P-DNA border sequence may be modified to increase the likelihood that backbone DNA from the accompanying vector is not integrated into the plant genome.

Table 2 below depicts the sequences of known T-DNA border sequences and sequences identified herein as border-like sequences. None of the sequences identified as "border-like" in Table 2 have been identified previously as having a T-DNA border-like structure. The potato border-like sequences were isolated by the present inventive methods using degenerate primers in polymerase chain reactions from potato genomic DNA. The present invention encompasses the use of any P-DNA border-like sequence for transferring a cojoined polynucleotide into the genome of a plant cell.

Indeed, the present invention encompasses any border-like sequence that has the nucleic acid sequence structure of SEQ ID NO. 93: ANGATNTATN6GT (SEQ ID NO. 93), where "N" is any nucleotide, such as those represented by "A," "G," "C," or "T." This sequence represents the consensus sequence of border-like nucleic acids identified by the present invention.

Table 2. "Border" and "Border-Like" sequences

***Agrobacterium* T-DNA borders**	
TGACAGGATATATTGGCGGGTAAAC (SEQ ID NO.41)	*Agrobacterium* nopaline strains (RB)
TGGCAGGATATATTGTGGTGTAAAC (SEQ ID NO.42)	*Agrobacterium* nopaline strains (LB)
TGGCAGGATATATACCGTTGTAATT (SEQ ID NO.43)	*Agrobacterium* octopine strains (RB)
CGGCAGGATATATTCAATTGTAATT (SEQ ID NO.44)	*Agrobacterium* octopine strains (LB)
TGGTAGGATATATACCGTTGTAATT (SEQ ID NO.45)	LB mutant
TGGCAGGATATATGGTACTGTAATT (SEQ ID NO.46)	LB mutant
YGRYAGGATATATWSNVBKGTAAWY (SEQ ID NO.47)	Border motif
Border-like sequences	
CGGCAGGATATATCCTGATGTAAAT (SEQ ID NO.48)	*R. leguminosarum*
TGGCAGGAGTTATTCGAGGGTAAAC (SEQ ID NO.49)	*T. tengcongensis*
TGACAGGATATATCGTGATGTCAAC (SEQ ID NO.50)	*Arabidopsis thaliana*
GGGAAGTACATATTGGCGGGTAAAC (SEQ ID NO.51)	*A. thaliana* CHR1v07142002
TTACAGGATATATTAATATGTATGA (SEQ ID NO.52)	*Oryza sativa* AC078894
TAACATGATATATTCCCTTGTAAAT (SEQ ID NO.53)	Homo sapiens clone HQ0089
TGACAGGATATATGGTAATGTAAAC (SEQ ID NO.54)	potato (left border sequence)*
TGGCAGGATATATACCGATGTAAAC (SEQ ID NO.55)	potato (right border sequence)*

Y= C or T; R= A or G; K= G or T; M= A or C; W= A or T; S= C or G; V= A, C, or G; B= C, G, or T. The accession numbers for the border-like sequences are: *Oryza sativa* chromosome 10 BAC OSJNBa0096G08 genomic sequence (AC078894.11); *Arabidopsis thaliana* chromosome 3 (NM_114337.1); *Arabidopsis thaliana* chromosome 1 (NM_105664.1); *T. tengcongensis* strain MB4T, section 118 of 244 of the complete genome (AE013091.1); Homo sapiens clone HQ0089 (AF090888.1); *Rhizobium* Clone: rhiz98e12.q1k. *potato left and right border sequences were obtained and isolated according to the presently-described inventive methods.

Carrier DNA: a "carrier DNA" is a DNA segment that is used to carry certain genetic elements and deliver them into a plant cell. In conventional foreign DNA transfer, this carrier DNA is often the T-DNA of *Agrobacterium*, delineated by border sequences. The carrier DNA described here is obtained from the selected plant species to be modified and contains ends that may be structurally and functionally different from T-DNA borders but shares with such T-DNAs the ability to support both DNA transfer from *Agrobacterium* to the nuclei of plant cells or certain other eukaryotes and the subsequent integration of this DNA into the genomes of such eukaryotes.

Consisting essentially of: a composition "consisting essentially of" certain elements is limited to the inclusion of those elements, as well as to those elements that do not materially affect the basic and novel characteristics of the inventive composition. Thus, so long as the composition does not affect the basic and novel characteristics of the instant invention, that is, does not contain foreign DNA that is not from the

selected plant species or a plant that is sexually compatible with the selected plant species, then that composition may be considered a component of an inventive composition that is characterized by "consisting essentially of" language.

Degenerate primer: a "degenerate primer" is an oligonucleotide that contains sufficient nucleotide variations that it can accommodate base mismatches when hybridized to sequences of similar, but not exact, homology.

Dicotyledon (dicot): a flowering plant whose embryos have two seed leaves or cotyledons. Examples of dicots include, but are not limited to, tobacco, tomato, potato, sweet potato, cassava, legumes including alfalfa and soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

Regulatory sequences: refers to those sequences which are standard and known to those in the art, that may be included in the expression vectors to increase and/or maximize transcription of a gene of interest or translation of the resulting RNA in a plant system. These include, but are not limited to, promoters, peptide export signal sequences, introns, polyadenylation, and transcription termination sites. Methods of modifying nucleic acid constructs to increase expression levels in plants are also generally known in the art (see, e.g. Rogers et al., 260 *J. Biol. Chem.* 3731-38, 1985; Cornejo et al., 23 *Plant Mol. Biol.* 567: 81,1993). In engineering a plant system to affect the rate of transcription of a protein, various factors known in the art, including regulatory sequences such as positively or negatively acting sequences, enhancers and silencers, as well as chromatin structure may have an impact. The present invention provides that at least one of these factors may be utilized in engineering plants to express a protein of interest. The regulatory sequences of the present invention are native genetic elements, i.e., are isolated from the selected plant species to be modified.

Foreign: "foreign," with respect to a nucleic acid, means that that nucleic acid is derived from non-plant organisms, or derived from a plant that is not the same species as the plant to be transformed or is not derived from a plant that is not interfertile with the plant to be transformed, does not belong to the species of the target plant.

According to the present invention, foreign DNA or RNA represents nucleic acids that are naturally occurring in the genetic makeup of fungi, bacteria, viruses, mammals, fish or birds, but are not naturally occurring in the plant that is to be transformed. Thus, a foreign nucleic acid is one that encodes, for instance, a polypeptide that is not naturally produced by the transformed plant. A foreign nucleic acid does not have to encode a protein product. According to the present invention, a desired transgenic plant is one that does not contain any foreign nucleic acids integrated into its genome.

Native genetic elements, on the other hand, can be incorporated and integrated into a selected plant species genome according to the present invention. Native genetic elements are isolated from plants that belong to the selected plant species or from plants that are sexually compatible with the selected plant species. For instance, native DNA incorporated into cultivated potato (*Solanum tuberosum*) can be derived from any genotype of *S. tuberosum* or any genotype of a wild potato species that is sexually compatible with *S. tuberosum* (*e.g.*, *S. demissum*).

Gene: "gene" refers to the coding region and does not include nucleotide sequences that are 5'- or 3'- to that region. A functional gene is the coding region operably linked to a promoter or terminator.

Genetic rearrangement: refers to the reassociation of genetic elements that can occur spontaneously *in vivo* as well as *in vitro* which introduce a new organization of genetic material. For instance, the splicing together of polynucleotides at different chromosomal loci, can occur spontaneously in vivo during both plant development and sexual recombination. Accordingly, recombination of genetic elements by non-natural genetic modification techniques in vitro is akin to recombination events that also can occur through sexual recombination in vivo.

In frame: nucleotide triplets (codons) are translated into a nascent amino acid sequence of the desired recombinant protein in a plant cell. Specifically, the present invention contemplates a first nucleic acid linked in reading frame to a second nucleic acid, wherein the first nucleotide sequence is a gene and the second nucleotide is a promoter or similar regulatory element.

Integrate: refers to the insertion of a nucleic acid sequence from a selected plant species, or from a plant that is from the same species as the selected plant, or from a plant that is sexually compatible with the selected plant species, into the genome of a cell of a selected plant species. "Integration" refers to the incorporation of only native genetic elements into a plant cell genome. In order to integrate a native genetic element, such as by homologous recombination, the present invention may "use" non-native DNA as a step in such a process. Thus, the present invention distinguishes between the "use of" a particular DNA molecule and the "integration" of a particular DNA molecule into a plant cell genome.

Introduction: as used herein, refers to the insertion of a nucleic acid sequence into a cell, by methods including infection, transfection, transformation or transduction.

Isolated: "isolated" refers to any nucleic acid or compound that is physically separated from its normal, native environment. The isolated material may be maintained in a suitable solution containing, for instance, a solvent, a buffer, an ion, or other component, and may be in purified, or unpurified, form.

Leader: Transcribed but not translated sequence preceding (or 5' to) a gene.

LifeSupport Vector: a LifeSupport vector is a construct that contains an expressable selectable marker gene, such as a neomycin phosphotransferase marker, that is positioned between T-DNA or T-DNA-like borders. The LifeSupport vector may be modified to limit integration of such a marker, as well as other polynucleotides, that are situated between the border or border-like sequences, into a plant genome. For instance, a LifeSupport vector may comprise a mutated virD2, *codA*::*upp* fusion, or any combination of such genetic elements. Thus, a modified virD2 protein will still support T-DNA transfer to plant nuclei but will limit the efficiency of a subsequent genomic integration of T-DNAs (Shurvinton et al., *Proc Natl Acad Sci USA,* 89: 11837-11841, 1992; Mysore et al., *Mol Plant Microbe Interact*, 11: 668-683, 1998). Alternatively, *codA*::*upp* gene fusion can be used as negative selectable marker prior to regeneration. In one preferred construct, the LifeSupport vector comprises the npt marker operably linked to the yeast ADH terminator element.

Monocotyledon (monocot): a flowering plant whose embryos have one cotyledon or seed leaf. Examples of monocots include, but are not limited to turf grass, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, and palm.

Native: a "native" genetic element refers to a nucleic acid that naturally exists in, orginates from, or belongs to the genome of a plant that is to be transformed. Thus, any nucleic acid, gene, polynucleotide, DNA, RNA, mRNA, or cDNA molecule that is isolated either from the genome of a plant or plant species that is to be transformed or is isolated from a plant or species that is sexually compatible or interfertile with the plant species that is to be transformed, is "native" to, *i.e.*, indigenous to, the plant species. In other words, a native genetic element represents all genetic material that is accessible to plant breeders for the improvement of plants through classical plant breeding. Any variants of a native nucleic acid also are considered "native" in accordance with the present invention. In this respect, a "native" nucleic acid may also be isolated from a plant or sexually compatible species thereof and modified or mutated so that the resultant variant is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% similar in nucleotide sequence to the unmodified, native nucleic acid isolated from a plant. A native nucleic acid variant may also be less than about 60%, less than about 55%, or less than about 50% similar in nucleotide sequence.

A "native" nucleic acid isolated from a plant may also encode a variant of the naturally occurring protein product transcribed and translated from that nucleic acid. Thus, a native nucleic acid may encode a protein that is greater than or equal to 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, 62%, 61%, or 60% similar in amino acid sequence to the unmodified, native protein expressed in the plant from which the nucleic acid was isolated.

Naturally occurring nucleic acid: this phrase means that the nucleic acid is found within the genome of a selected plant species and may be a DNA molecule or an RNA molecule. The sequence of a restriction site that is normally present in the genome of a plant species can be engineered into an exogenous DNA molecule, such as a vector or oligonucleotide, even though that restriction site was not physically isolated from that genome. Thus, the present invention permits the synthetic creation of a nucleotide sequence, such as a restriction enzyme recognition sequence, so long as that sequence is naturally occurring in the genome of the selected plant species or in a plant that is sexually compatible with the selected plant species that is to be transformed.

Operably linked: combining two or more molecules in such a fashion that in combination they function properly in a plant cell. For instance, a promoter is operably linked to a structural gene when the promoter controls transcription of the structural gene.

P-DNA: according to the present invention, P-DNA ("plant-DNA") is isolated from a plant genome and comprises at each end, or at only one end, a T-DNA border-like sequence. The border-like sequence preferably shares at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 90% or at least 95%, but less than 100% sequence identity, with a T-DNA border sequence from an *Agrobacterium* species, such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Thus, P-DNAs can be used instead of T-DNAs to transfer a nucleotide sequence from *Agrobacterium* to another polynucleotide sequence. The P-DNA may be modified to facilitate cloning and should preferably not naturally encode proteins or parts of proteins. The P-DNA is characterized in that it contains, at each end, at least one border sequence, referred to as either a "P-DNA border sequence" or "P-DNA border-like sequence," which are interexchangeable terms. See the definition of a "border sequence" and "border-like" above. A P-DNA may also be regarded as a "T-DNA-like" sequence, see definition below.

Plant: includes angiosperms and gymnosperms such as potato, tomato, tobacco, alfalfa, lettuce, carrot, strawberry, sugarbeet, cassava, sweet potato, soybean, maize,

turf grass, wheat, rice, barley, sorghum, oat, oak, eucalyptus, walnut, and palm. Thus, a plant may be a monocot or a dicot. The word "plant," as used herein, also encompasses plant cells, seed, plant progeny, propagule whether generated sexually or asexually, and descendents of any of these, such as cuttings or seed.. Plant cells include suspension cultures, callus, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, seeds and microspores. Plants may be at various stages of maturity and may be grown in liquid or solid culture, or in soil or suitable media in pots, greenhouses or fields. Expression of an introduced leader, trailer or gene sequences in plants may be transient or permanent. , A "selected plant species" may be, but is not limited to, a species of any one of these "plants."

Precise breeding: refers to the improvement of plants by stable introduction of nucleic acids, such as native genes and regulatory elements isolated from the selected plant species, or from another plant in the same species as the selected plant, or from species that are sexually compatible with the selected plant species, into individual plant cells, and subsequent regeneration of these genetically modified plant cells into whole plants. Since no unknown or foreign nucleic acid is permanently incorporated into the plant genome, the inventive technology makes use of the same genetic material that is also accessible through conventional plant breeding.

Plant species: the group of plants belonging to various officially named plant species that display at least some sexual compatibility.

Plant transformation and cell culture: broadly refers to the process by which plant cells are genetically modified and transferred to an appropriate plant culture medium for maintenance, further growth, and/or further development.

Recombinant: as used herein, broadly describes various technologies whereby genes can be cloned, DNA can be sequenced, and protein products can be produced. As used herein, the term also describes proteins that have been produced following the transfer of genes into the cells of plant host systems.

Selectable marker: a "selectable marker" is typically a gene that codes for a protein that confers some kind of resistance to an antibiotic, herbicide or toxic compound, and is used to identify transformation events. Examples of selectable markers include the streptomycin phosphotransferase (*spt*) gene encoding streptomycin resistance, the phosphomannose isomerase (*pmi*) gene that converts mannose-6-phosphate into fructose-6 phosphate; the neomycin phosphotransferase (*nptII*) gene encoding kanamycin and geneticin resistance, the hygromycin phosphotransferase (*hpt* or *aphiv*) gene encoding resistance to hygromycin, acetolactate synthase (*als*) genes encoding resistance to sulfonylurea-type herbicides, genes coding for resistance to herbicides which act to inhibit the action of glutamine synthase such as phosphinothricin or basta (e.g., the bar gene), or other similar genes known in the art.

Sense suppression: reduction in expression of an endogenous gene by expression of one or more an additional copies of all or part of that gene in transgenic plants.

T-DNA-Like: a "T-DNA-like" sequence is a nucleic acid that is isolated from a selected plant species, or from a plant that is sexually compatible with the selected plant species, and which shares at least 75%, 80%, 85%, 90%, or 95%, but not 100%, sequence identity with *Agrobacterium* species T-DNA. The T-DNA-like sequence may contain one or more border or border-like sequences that are each capable of integrating a nucleotide sequence into another polynucleotide. A "P-DNA," as used herein, is an example of a T-DNA-like sequence.

Trailer: Transcribed but not translated sequence following (or 3'to) a gene.

Transcribed DNA: DNA comprising both a gene and the untranslated leader and trailer sequence that are associated with that gene, which is transcribed as a single mRNA by the action of the preceding promoter.

Transcription and translation terminators: the expression vectors of the present invention typically have a transcription termination region at the opposite end from the transcription initiation regulatory region. The transcription termination region may be selected, for stability of the mRNA to enhance expression and/or for the addition of polyadenylation tails added to the gene transcription product (Alber &

Kawasaki, *Mol. & Appl. Genetics* 4: 19-34, 1982). Illustrative transcription termination regions include the E9 sequence of the pea *RBCS* gene (Mogen et al., *Mol. Cell Biol.*, 12: 5406-14, 1992) and the termination signals of various ubiquitin genes.

Transformation of plant cells: a process by which DNA is stably integrated into the genome of a plant cell. "Stably" refers to the permanent, or non-transient retention and/or expression of a polynucleotide in and by a cell genome. Thus, a stably integrated polynucleotide is one that is a fixture within a transformed cell genome and can be replicated and propagated through successive progeny of the cell or resultant transformed plant. Transformation may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of nucleic acid sequences into a prokaryotic or eukaryotic host cell, including *Agrobacterium*-mediated transformation protocols, viral infection, whiskers, electroporation, heat shock, lipofection, polyethylene glycol treatment, micro-injection, and particle bombardment.

Transgene: a gene that will be inserted into a host genome, comprising a protein coding region. In the context of the instant invention, the elements comprising the transgene are isolated from the host genome.

Transgenic plant: a genetically modified plant which contains at least one transgene.

Using/Use of: The present invention envisions the use of nucleic acid from species other than that of the selected plant species to be transformed to facilitate the integration of native genetic elements into a selected plant genome, so long as such foreign nucleic acid is not stably integrated into the same host plant genome. For instance, the plasmid, vector or cloning construct into which native genetic elements are cloned, positioned or manipulated may be derived from a species different to that from which the native genetic elements were derived.

Variant: a "variant," as used herein, is understood to mean a nucleotide or amino acid sequence that deviates from the standard, or given, nucleotide or amino acid sequence of a particular gene or protein. The terms, "isoform," "isotype," and

"analog" also refer to "variant" forms of a nucleotide or an amino acid sequence. An amino acid sequence that is altered by the addition, removal or substitution of one or more amino acids, or a change in nucleotide sequence, may be considered a "variant" sequence. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. A variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted may be found using computer programs well known in the art such as Vector NTI Suite (InforMax, MD) software.

It is understood that the present invention is not limited to the particular methodology, protocols, vectors, and reagents, etc., described herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a gene" is a reference to one or more genes and includes equivalents thereof known to those skilled in the art and so forth. Indeed, one skilled in the art can use the methods described herein to express any native gene (known presently or subsequently) in plant host systems.

P-DNA vectors

Agrobacterium-mediated transformation methods are the preferred means of incorporating recombined DNA into plant cells. According to the present invention, a binary vector was developed to produce genetically modified potato plants that contain only native potato nucleic acids. Such a vector is different from conventional, *Agrobacterium*-mediated transformation vectors in three ways: (1) instead of an *Agrobacterium*-derived T-DNA sequence delineated by T-DNA borders, the present vector contains a native plant DNA (P-DNA) fragment that is flanked by border-like sequences, which support P-DNA transfer from *Agrobacterium* to plant cells although they are structurally and functionally different from T-DNA borders, (2) the backbone

of the present vector may contain a marker that, if integrated into the plant cell's genome, prevents these cells from developing into mature plants, and (3) the present vector does not contain a foreign selectable marker gene between P-DNA termini.

The present invention demonstrates, surprisingly, that P-DNA fragments flanked by border-like sequences support DNA transfer from *Agrobacterium* into plant cells. P-DNA can be isolated from the genome of any plant by using primers that are designed on the basis of homology between the termini of a potato P-DNA and conventional T-DNA borders. Such fragments can then be tested and, if efficacious, used to transform that plant with native DNA exclusively. It is also possible to search plant genomic databases for DNA fragments with regions that show homology with T-DNA borders by using programs such as 'blastn' (Altschul et al., *J Mol Biol* 215: 403-10, 1990). The identified P-DNAs may then be modified to increase their utility. For instance, internal fragments of the isolated P-DNAs may be deleted and restriction sites may be added to facilitate cloning. It may also be efficacious to introduce point mutations at the terminal sequences to render the P-DNA more effective in transferring DNA.

Any gene expression cassette can be inserted between P-DNA border-like sequences. For potato transformations, such an expression cassette could consist of a potato promoter, operably linked to a potato gene and/or a leader or trailer sequence associated with that gene, and followed by a potato terminator. The expression cassette may contain additional potato genetic elements such as a signal peptide sequence fused in frame to the 5'-end of the gene, and a potato intron that could, for instance, be placed between promoter and gene-of-interest to enhance expression. For transformation of wheat with a modified P-DNA, all genetic elements that are inserted on the wheat P-DNA, including the P-DNA itself would be derived from wheat or plant species that are sexually compatible with wheat.

Another way to isolate P-DNAs is by generating a library of *Agrobacterium* strains that contain random plant DNA fragments instead of a T-DNA flanking a selectable marker gene. Explants infected with this library can be placed on proliferation

medium that contains an appropriate selectable agent to identify P-DNAs that support the transfer of the marker gene from the vector in *Agrobacterium* to the plant cell.

It is possible that not just the native modified P-DNA, but also additional plasmid sequences are co-transferred from *Agrobacterium* to the plant cell during the transformation process. For the purposes of the present invention, this is an undesirable process because such plasmid "backbone" sequences represent non-plant, foreign DNA, such as bacterial DNA. The present invention prevents transformed plant cells that contain backbone sequences from developing into mature plants. Thus, the present invention makes it possible to distinguish backbone-containing and backbone-free transformation events during the regenerated shoot phase.

The method to select or screen against backbone integration events relies on the presence of an expression cassette for a marker, such as the isopentenyl phosphotransferase (IPT) gene, in the vector backbone, outside of the P-DNA. Upon backbone integration, the accumulation of IPT-induced cytokinin will alter the shape of transformed shoots, and prevent these shoots to develop roots. Instead of the IPT gene, any other gene that alters the shape, texture or color of the transformed plant's leaves, roots, stem, height or some other morphological feature can be used to screen and/or select against backbone integration events. Such a gene is referred to herein as a "backbone integration marker." Thus, the transformed plant that exhibits an altered morphological feature attributable to the expression of the backbone integration marker gene is known to, contain in its genome foreign DNA in addition to the desired P-DNA. Accordingly, plants that exhibit a phenotype associated with the backbone integration marker are not desired.

The present invention is not limited to the use of only an IPT gene as a backbone integration marker; other genes can be used in such fashion. For example, a backbone integration marker may be an *Agrobacterium* transzeatine synthase (*TZS*) gene (Krall et al., *FEBS Lett* 527: 315-8, 2002) or a recessive *Arabidopsis* gene hoc1 (Catterou et al., *Plant J* 30: 273-87, 2002). This method can be more easily applied for use in the present invention than some methods that insert toxic genes in vector backbone sequences. See, for instance,EP 1 009,842.

By positioning a backbone integration marker gene, such as a functional cytokinin gene upstream or downstream of the P-DNA, it is straightforward to distinguish between transformation events. Transformed plants that exhibit an altered morphological feature are discarded because they contain non-native DNA sequences integrated into the genome.

Another strategy for identifying plants that are stably transformed with only native DNA, is to employ the polymerase chain reaction. By using primers that are specifically designed to detect backbone sequences, plants can be identified and discarded that contain foreign backbone sequences in addition to the P-DNA. Other primer sets can subsequently be used to confirm the intact transfer of the P-DNA. Thus, by either using the expression of a gene to change a morphological feature of a plant, or by screening for stably integrated foreign DNA in a transformed plant, plants stably transformed with only native DNA sequences can be identified and selected.

Genetic elements from a particular host plant can be inserted into the P-DNA sequence of a binary vector capable of replication in both *E. coli* and *Agrobacterium*. Introduction of the resulting vectors into disarmed *Agrobacterium* strains such as LBA4404 can be accomplished through electroporation, triparental mating or heat-shock treatment of chemically competent cells. The new strains can then be used to transform individual plant cells through infection of whole plants or explants.

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LifeSupport vectors

Although the stable integration of bacterial marker genes into the genomes of plant cells facilitates the identification of transformation events, such modifications of plant genomes are undesirable because marker genes represent foreign DNA. Use of a foreign marker gene can be avoided by developing new *Agrobacterium*-based transformation methods.

One preferred embodiment is a novel method that relies on the use of two *Agrobacterium* strains: one strain containing a binary vector with a selectable marker gene intended for transient expression in plant nuclei, and another strain carrying the P-DNA with the actual sequences of interest intended for stable integration in plant genome (see Example 7).

Upon co-infection with the *Agrobacterium* strains, some plant cells will receive both a T-DNA with the marker gene and a P-DNA with the sequences of interest. Instead of subsequently selecting for stable integration of the marker gene by subjecting the infected explants for a long period of time to the appropriate antibiotic, explants are only briefly exposed to the antibiotic. In this way, all plant cells that transiently express the marker gene will survive. Because T-DNAs will in most cases degrade due to endogenous nuclease activities rather than stably integrate into their host's genome, the majority of plant cells that survived the transient selection are shown here to develop into shoots lacking a marker gene. The present invention, furthermore, demonstrates that a significant proportion of these marker-free shoots contain stably integrated P-DNAs.

There are various tools to enhance the efficiency of marker-free transformation. First, the present invention demonstrates that this frequency can be increased by sequentially infecting explants with two *Agrobacterium* strains carrying the T-DNA/marker and P-DNA/sequences-of-interest, respectively. Explants are first infected with the P-DNA strain, and after about 4 to 6 hours with the T-DNA strain.

Second, the T-DNA strain can be modified to express an omega-mutated virD2 gene. The modified virD2 protein will still support T-DNA transfer to plant nuclei but limit

the efficiency of a subsequent genomic integration of T-DNAs (Shurvinton et al., *Proc Natl Acad Sci USA,* 89: 11837-11841, 1992; Mysore et al., *Mol Plant Microbe Interact*, 11: 668-683, 1998). The most preferred method of expressing a modified *virD2* gene is by inserting an omega-mutated *virD2* gene driven by the virD promoter in the backbone of the T-DNA vector.

Third, stable T-DNA integration can be further impaired by inserting telomere sequences close to the left- and right- border sequences of the T-DNA (Chiurazzi & Signer, Plant Mol. Biol., 26: 923-934, 1994).

Fourth, the size of the T-DNA region carrying the marker gene can be increased to enhance the frequency of T-DNAs and P-DNAs moving together into the plant cell nucleus, and to reduce the frequency of genomic integration of the T-DNA.

Fifth, the frequency of T-DNAs and P-DNAs moving together into the plant cell nucleus can also be enhanced by using a single *Agrobacterium* strain carrying two compatible binary vectors with the T-DNA and P-DNA, respectively. An example of two compatible binary vectors are a pSIM 1301-derived vector and a pBI121-derived vector.

Because the transiently expressed marker gene will usually not integrate into the plant genome, it is not necessary that both this gene and its regulatory sequences represent native DNA. In fact, it may be advantageous to use foreign regulatory sequences to promote high levels of transient gene expression in infected plant cells. A surprising discovery of the present invention is that an expression cassette containing the GUS gene followed by the terminator of the yeast alcohol dehydrogenase 1 (*ADH1*) was transiently expressed at high levels in potato cells. A similar construct with the yeast *CYC1* terminator, however, did not function adequately. It may also be possible to enhance transient expression levels by operably linking a marker gene to a non-native promoter. Examples of such promoters are, e.g., synthetic promoters such as glucocorticoid-inducible promoters (Mori et al., Plant J., 27: 79-86, 2001; Bohner et al., Mol. Gen. Genet., 264: 860-70 2001), and non-native promoters such as the 35S

promoters of cauliflower mosaic virus and figwort mosaic virus, and fungal promoters.

As an alternative to the two-strain *Agrobacterium*-mediated transformation approach described above, plants may also be transformed with a single strain that contains a P-DNA with both a native marker gene and the actual sequences of interest. The present invention demonstrates that it is possible to use salt tolerance genes as native markers for transformation. Such salt tolerance genes include crop homologs of the *Arabidopsis* genes *SOS1* (Shi et al., *Nat Biotechnol.* 2002), *AtNHX1* (Apse et al., *Science*. 285: 1256-8, 1999), *Avp1* (Gaxiola et al., *Proc Natl Acad Sci U S A*. 98: 11444-9, 2001), and *CBF3* (Kasuga et al., *Nat Biotechnol.* 17: 287-91, 1999).

The rearrangements of genetic elements accomplished through the inventive Precise Breeding methodology could also occur spontaneously through the process of genetic recombination. For instance, all plants contain elements that can transpose from one to another chromosomal location. By inserting into promoters or genes, such transposable elements can enhance, alter, and/or reduce gene expression. For instance, the AMu4 insertion of the maize *Mutator* element in the promoter of the transcriptional regulator gene *P-wr* causes stripy red pericarps. Insertion of the same element in the promoter of the leaf-specific MADS-box gene *ZMM19* resulted in expression of this gene in the inflorescences of maize, causing a foliaceous elongation of the glumes and other changes in male and female inflorescences, resulting in the famous phenotype of pod corn. Because of its bizarre tassels and ears, pod corn was of religious significance for certain native American tribes. Many genes are also rearranged through other transposon-induced modifications such as inversions, deletions, additions, and ectopic recombinations (Bennetzen, *Plant Mol Biol* 42: 251-69, 2000). Furthermore, plant DNA rearrangements frequently occur through the process of intragenic recombination. For instance, by recombining genes involved in resistance against specific pathogens, plants are able to develop resistance genes with new specificities and, thus, co-evolve with their pathogens (Ellis et al., *Trends Plant Sci* 5: 373-9, 2000). Another example of intragenic recombination relates to how plants reproduce: plants transition from cross-fertilizing to self-fertilizing by

recombining genes involved in self-incompatibility (Kusaba et al., *Plant Cell* 13: 627-43, 2001). Other processes that promote genome evolution include, for instance, chromosome breakage and interchromosomal recombination.

Enhancing the nutritional value of plants and food crops

To modify negative traits such as acrylamide accumulation during processing, glycoalkaloid accumulation, accumulation of undesirable advanced glycation products, CIPC accumulation, low levels of resistant starch, bruise susceptibility, cold-induced sweetening, disease susceptibility, low yield and low quality in crop plants through precise breeding, at least one specific expression cassette is incorporated into a host genome. Three different methods are used to eliminate negative traits: (1) overexpression of genes that prevent the occurrence of negative traits, (2) overexpression of mutated versions of genes associated with negative traits in order to titrate out the wild-type gene products with non-functional proteins, and (3) silencing specific genes that are associated with a negative trait by expressing at least one copy of a leader or trailer fragment associated with that gene in the sense and/or antisense orientation.

One example of an endogenous gene that is associated with a negative trait in potato and can be modified *in vitro* so that it encodes a non-functional protein is the polyphenol oxidase (*PPO*) gene. Upon impact injury, the *PPO* gene product is released from the plastid into the cytoplasm (Koussevitzky et al., *J. Biol. Chem.*, 273: 27064-9, 1998), where it will mediate the oxidation of phenols to create a variety of phenoxyl radicals and quinoid derivatives, which are toxic and/or ultimately form undesirable polymers that leave dark discolorations, or "black spots" in the crop.

Overexpressing a mutant *PPO* gene that contains a non-functional copper-binding domain can lower the activity of all *PPO* genes that are mainly expressed in tubers and associated organs such as sprouts. The mutations render the polyphenol oxidase protein inactive because it is unable to bind copper. The skilled artisan would know where to make point mutations that would, in this case, compromise the function of a gene product. The applicants identified the copper binding domain in potato *PPO* by

aligning the potato PPO protein sequence with a sweet potato PPO protein sequence (Klabunde et al., Nat Struct. Biol., 5:1084-90, 1998). Areas of conservation, particularly those containing conserved histidine residues in copper-binding sites, were targets for inactivating the transgene product. Because the almost complete absence of PPO activity in such organs may negatively impact the plant's ability to resist pathogens, the present invention also describes an improved method of only lowering a specific *PPO* gene that is predominantly expressed in all parts of the mature tuber except for the epidermis. Silencing of this specific *PPO* gene by using a trailer sequence associated with that gene does not reduce *PPO* expression in the tuber epidermis, the part of the tuber that is most directly exposed to pathogens attempting to infect.

Enzymatic browning induced by the *PPO* gene not only reduces the quality of potato tubers; it also negatively affects crop foods such as wheat, avocado, banana, lettuce, apple, and pears.

Other genes that are associated with negative traits and can be silenced by using the leader or trailer sequences associated with those genes include the potato *R1* gene and *L-type phosphorylase* genes. Both genes are involved in the degradation of starch to reducing sugars, such as glucose and fructose, which upon heating participate in the Maillard reaction to produce toxic products such as acrylamide. The present invention demonstrates that a reduction of cold-induced sweetening by lowering *R1* or phosphorylase activity leads to a reduction of both non-enzymatic browning and acrylamide accumulation during the frying process of potatoes.

The invention also demonstrates the utility of overexpressing certain native genes in genetically modified crops. Levels of Maillard-reaction products such as acrylamide were reduced significantly by lowering the conversion of sucrose to reducing sugars through overexpression of a newly isolated vacuolar invertase inhibitor gene in potato.

The present invention also predicts that potato tubers displaying either an increased level of invertase inhibitor expression or a reduced level of *R1* or phosphorylase

expression will not require the intensive treatment with chemical sprout inhibitors such as CIPC prior to storage because their lowered levels of reducing sugars will (1) delay sprouting, and (2) allow storage at lower temperatures, thus further delaying sprouting. The highly reduced CIPC-residue levels, or the absence thereof, further enhances the nutritional value of processed foods derived from plants containing certain modified P-DNAs described here.

Thus, French fries or chips derived from tubers that contain the modified P-DNA will contain strongly reduced CIPC residue levels, further boosting their nutritional value.

The effect of simultaneously downregulating the expression of the *PPO* and either *R1* or phosphorylase genes in potato tubers is synergistic because reducing sugars are not only required for non-enzymatic browning through the Maillard reaction but also for browning mediated by the PPO enzyme. Decreased levels of reducing sugars in transgenic potato tubers will, therefore, also limit PPO activity and black spot bruise susceptibility. Thus, *PPO*, *R1*, and phosphorylase genes, and/or the leader or trailer sequences that are associated with these genes, represent DNA segments of interest that can be isolated, modified and reintroduced back into the plant to down-regulate the expression of these genes.

Apart from developing bruise resistance and reduced cold-induced sweetening, there are many other traits that can be introduced through Precise Breeding without using foreign DNA. For instance, disease resistance genes can be isolated from wild potato species and inserted into the genomes of disease susceptible varieties.

The environmental benefits of modified plants and crops

As described above, reduced levels of either *R1* or *phosphorylase* result in a reduced phosphorylation of starch. This reduction in starch phosphorylation results in a 90% decrease in phosphate content of potato tubers (Vikso-Nielsen, *Biomacromolecules*, 2: 836-43, 2001). This will result in a reduction in phosphate levels in wastewaters from potato processing plants, which are currently about 25-40 mg/L. Thus, the use of low-phosphate tubers will reduce the release of phosphates into the environment and help to protect important ecosystems. Furthermore, low-phosphate potatoes may

require less phosphate fertilization for optimal growth and yield, which would support a more sustainable agriculture by delaying the depletion of available phosphate resources.

Enhancing the agricultural performance of plants and food crops

Apart from reduced bruise susceptibility and reduced cold-sweetening, which are two important processing traits, the present invention also provides salt tolerance, an increasingly important input trait. Some of the modified P-DNA constructs described in the present invention contain a salt tolerance gene as native marker for transformation. Importantly, the utility of this gene is not limited to a screening step in the transformation procedure. Overexpression of the salt tolerance gene in potato plants reduces stress symptoms induced by high salinity soil levels, and will make it possible to grow new varieties containing a modified P-DNA on a growing percentage of agricultural lands that contain salinity levels exceeding the maximum 2 millimhos/cm electrical conductivity levels that are optimal for growing conventional varieties.

Using regulatory elements isolated from a selected plant species or from a species sexually compatible with the selected plant species

Once the leader, gene or trailer has been isolated from the plant species of interest, and optionally modified, it can be operably linked to a plant promoter or similar regulatory element for appropriate expression in plants. Regulatory elements such as these serve to express untranslated sequences associated with a gene of interest in specific tissues or at certain levels or at particular times.

Dependent on the strategy involved in modifying the trait, it may be necessary to limit silencing to a particular region of the plant. The promoter normally driving the expression of the endogenous gene may not be suitable for tissue-specific expression. As described in the section above, stable integration of bacterial or viral regulatory components, such as the cauliflower mosaic virus 35S "super" promoter, can result in unpredictable and undesirable events. Thus, one aspect of the present invention uses promoters that are isolated from the selected host plant species.

In a preferred embodiment of the instant invention, for use in *S. tuberosum*, the leader or trailer sequences associated with *R1*, phosphorylase, and *PPO* genes are operably linked to the granule-bound starch synthase gene promoter (Rohde et al., *J Gen & Breed*, 44, 311-315, 1990). This promoter has been used frequently by others to drive gene expression and is particularly active in potato tubers (van der Steege et al., *Plant Mol Biol*, 20: 19-30, 1992; Beaujean et al., *Biotechnol. Bioeng*, 70: 9-16, 2000; Oxenboll et al., *Proc Natl Acad Sci USA*, 9: 7639-44, 2000). This promoter may also be used, in a preferred embodiment, for expression of the modified leader or trailer sequences of *R1*, phosphorylase, and *PPO* genes.

Alternatively, other potato promoters can be operably linked to sequences of interest from potato. Such promoters include the patatin gene promoter (Bevan et al., *Nucleic Acids Res*, 14: 4625-38, 1986), or a fragment thereof, that promotes expression in potato tubers, the potato UDP-glucose pyrophosphorylase gene promoter (U.S. Patent No. 5,932,783) and the promoter of the ubiquitin gene (Garbarino et al., *Plant Physiol*, 109: 1371-8, 1995).

The transcription of leaders and/or trailers can also be regulated by using inducible promoters and regulatory regions that are operably linked in a construct to a polynucleotide of interest. Examples of inducible promoters include those that are sensitive to temperature, such as heat or cold shock promoters. For instance, the potato ci21A-, and C17- promoters are cold-inducible (Kirch et al., *Plant Mol. Biol*, 33: 897-909, 1997; Schneider et al., *Plant Physiol*, 113: 335-45, 1997).

Other inducible promoters may be used that are responsive to certain substrates like antibiotics, other chemical substances, or pH. For instance, abscisic acid and gibberellic acid are known to affect the intracellular pH of plant cells and in so doing, regulate the Rab 16A gene and the alpha-amylase 1/6-4 promoter (Heimovaara-Dijkstra *et al.*, *Plant Mol Biol*, 4 815-20, 1995). Abscisic acid, wounding and methyl jasmonate are also known to induce the potato pin2 promoter (Lorberth et al., *Plant J*, 2: 477-86, 1992).

In another example, some nucleotide sequences are under temporal regulation and are activated to express a downstream sequence only during a certain developmental stage of the plant or during certain hours of the day. For instance, the potato promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*) gene can direct cell-specific, light-regulated expression (Fritz et al., *Proc Natl Acad Sci USA*, 88: 4458-62, 1991). The skilled artisan is well versed in these exemplary forms of inducible promoters and regulatory sequences.

The use of certain polyadenylation signals may also be useful in regulating expression, by varying the stability of the mRNA transcript. In particular, some polyadenylation signals when operably linked to the '3 end of a polynucleotide cause the mRNA transcript to become accessible to degradation.

Thus, it is possible to regulate expression of a gene by operably linking it with one or more of such promoters, regulatory sequences, 3' polyadenylation signals, 3' untranslated regions, signal peptides and the like. According to the instant invention, DNA sequences and regulatory elements such as those described herein, and which will ultimately be integrated into a plant genome, are obtained from DNA of the selected plant species to be modified by the Precise Breeding process of the present invention. That is, DNA sequences and regulatory elements that are derived, isolated and cloned from other species, such as from bacteria, viruses, microorganisms, mammals, birds, reptiles and sexually incompatible plant species are not integrated into the genome of the transformed plant. DNA foreign to the selected plant species genome may be used in the present invention to create a transformation construct, so long as that foreign DNA is not integrated into a plant genome.

Not only does the present invention provide a method for transforming a plant species by integrating DNA obtained from the selected plant species, or from a plant that is sexually-compatible with the selected plant species, it also provides a means by which the expression of that DNA can be regulated. Accordingly, it is possible to optimize the expression of a certain sequence, either by tissue-specific or some other strategy, as previously described.

Using 3' terminator sequences isolated from a selected plant species

In addition to regulatory elements that initiate transcription, the native expression cassette also requires elements that terminate transcription at the 3'-end from the transcription initiation regulatory region. The transcription termination region and the transcription initiation region may be obtained from the same gene or from different genes. The transcription termination region may be selected, particularly for stability of the mRNA to enhance expression.

This particular element, the so-called "3'-untranslated region" is important in transporting, stabilizing, localizing and terminating the gene transcript. In this respect, it is well known to those in the art, that the 3'-untranslated region can form certain hairpin loop. Accordingly, the present invention envisions the possibility of operably linking a 3' untranslated region to the 3' end of a cloned polynucleotide such that the resultant mRNA transcript may be exposed to factors which act upon sequences and structures conferred by the 3' untranslated region.

A 3' sequence of the ubiquitin gene can be subcloned from the plant species from which the promoter and transgene were isolated and inserted downstream from a transgene to ensure appropriate termination of transcription. Both exemplary transgenes can be fused to the terminator sequence of the potato Ubiquitin gene (Ubi3) regardless of which promoter is used to drive their expression.

EXAMPLES

Example 1

Cloning of P-DNAs

This example demonstrates that T-DNA borders are specific to *Agrobacterium*. It also shows that plants contain T-DNA border-like sequences, and it provides the sequence of DNA fragments isolated from potato and wheat that are delineated by such border-like sequences.

Conventional transformation systems use *Agrobacterium*-derived T-DNAs as vehicles for the transfer of foreign DNA from *Agrobacterium* to plant cells (Schilperoort et al., *US Patent* 4940838, 1990). Although T-DNAs usually comprise several hundreds of basepairs, delineated by a left-border (LB) and right-border (RB) repeat, they can also merely consist of such borders. The T-DNA borders play an essential role in the DNA transfer process because they function as specific recognition sites for virD2-catalyzed nicking reaction. The released single stranded DNA, complexed with Agrobacterial virD2 and virE2, is transferred to plant cell nuclei where it often integrates successfully into the plant genome. All T-DNA borders that have been used for foreign DNA transfer are derived from nopaline and octopine strains of *Agrobacterium tumefaciens* and *A. rhizogenes* (Table 2). These borders and often some flanking *Agrobacterium* DNA are present in thousands of binary vectors including, for example, pPAM (AY027531), pJawohl (AF408413), pYL156 (AF406991), pINDEX (AF294982), pC1300 (AF294978), pBI121 (AF485783), pLH9000 (AF458478), pAC161 (AJ315956), BinHygTOp (Z37515), pHELLSGATE (AJ311874), pBAR-35S (AJ251014), pGreen (AJ007829), pBIN19 (X77672), pCAMBIA (AF354046), pX6-GFP (AF330636), pER8 (AF309825), pBI101 (U12639), pSKI074 (AF218466), pAJ1 (AC138659), pAC161 (AJ315956), pSLJ8313 (Y18556), and pGV4939 (AY147202). Recently, two homologs of T-DNA borders were identified in the chrysopine-type Ti plasmid pTiChry5 (Palanichelvam et al., *Mol Plant Microbe Interact* 13: 1081-91, 2000). The left border homolog is identical to an inactive border homolog located in the middle of the T-DNA of pTi15955. The right border homolog is unusually divergent from the sequence of functional T-DNA borders. It is therefore unlikely that these homologs are functionally active in supporting DNA transfer from pTiChry5 to plant cells.

Development of a new method that makes it possible to transform plants with only native DNA requires, in the first place, a replacement of the T-DNA including LB and RB. Unfortunately, advanced BLAST searches of public databases including those maintained by The National Center For Biotechnology Information, The Institute for Genomic Research, and SANGER failed to identify any border sequences in plants. It was therefore necessary to consider plant DNA sequences that are similar but not

identical to T-DNA borders, designated here as "border-like" (border-like). Examples of plant border-like sequences that were identified in public databases are shown in Table 2. The challenge in trying to replace T-DNA borders with border-like sequences is that border sequences are highly conserved (see Table 2). A large part of these sequences is also highly conserved in the nick regions of other bacterial DNA transfer systems such as that of IncP, PC194, and ϕX174, indicating that these sequences are essential for conjugative-like DNA transfer (Waters et al., *Proc Natl Acad Sci* 88: 1456-60, 1991). Because there are no reliable data on border sequence requirements, the entire border seems therefore important in the nicking process. A single study that attempted to address this issue by testing the efficacy of border mutants in supporting DNA transfer is unreliable because negative controls did not appear to function appropriately (van Haaren et al., *Plant Mol Biol* 13: 523-531, 1989). Furthermore, none of the results of this study were confirmed molecularly. Despite these concerns, two possibly effective border mutants are shown in Table 2 as well.

Based on the homology among border sequences, a T-DNA border motif was identified (Table 2). Although this motif comprises 13,824 variants, many of which may not function –or may be inadequate- in transferring DNA, it represents the broadest possible definition of what a T-DNA border sequence is or may be. This border motif was then used to search publicly available DNA databases for homologs using the "Motif Alignment and Search Tool" (Bailey and Gribskov, *Bioinformatics* 14: 48-54, 1998) and "advanced BLASTN" ("penalty for nucleotide mismatch" = -1; "expect" = 10^5; Altschul et al., *Nucleic Acids Res* 25: 3389-3402, 1997). Again, these searches did not identify any identical matches in organisms other than *Agrobacterium*.

To try and increase the chance of isolating a potato DNA fragment containing border-like sequences that correspond to the border motif, DNA was isolated from 100 genetically diverse accessions (the so-called "core collection," provided by the US Potato Genebank, WI). This DNA was pooled and used as template for polymerase chain reactions using a variety of oligonucleotides designed to anneal to borders or

border-like sequences. Amplified fragments were sequence analyzed, and the sequence was then confirmed using inverse PCR with nested primers. One of the potato DNA fragments that was of particular interest contains a novel sequence without any major open reading frames that is delineated by border-like sequences (Table 2). One of the border-like sequences of this fragment contains at least 5 mismatches with T-DNA borders; the other border-like sequence contains at least 2 mismatches. Although both sequences contain one mismatch with the border motif, they were tested for their ability to support DNA transfer. For that purpose, the fragment was first reduced in size to 0.4-kilo basepairs by carrying out an internal deletion (SEQ ID NO.: 1). The resulting fragment was designated "P-DNA" (plant DNA) to distinguish it from the *Agrobacterium*-derived T-DNA. A similar fragment was isolated from the genome of the potato variety Russet Ranger, but has not been used for any further experiments.

Based on the divergence between P–DNA and T-DNA borders, the elongase amplification system (Life Technologies) was used with the following degenerate primers to isolate a P-DNA from wheat: 5'-GTTTACANHNBNATATATCCTGYCA –3' (Bor-F) (SEQ ID NO. 56), and 5'-TGRCAGGATATATNVNDNTGTAAAC –3' (Bor-R) (SEQ ID NO. 57). The resulting 825-bp fragment is shown in SEQ ID NO.: 2, and was used to replace the T-DNA of a conventional binary vector. The efficacy of this construct can be tested by inserting an expression cassette for the GUS gene between P-DNA termini, and infecting wheat with an *Agrobacterium* strain carrying the resulting vector.

Example 2

Tobacco transformation with P-DNA vectors

This Example demonstrates that, despite structural (sequence divergence) and functional (transformation frequencies) differences between P-DNA termini and T-DNA borders, a P-DNA can be used in a similar way as a T-DNA to transfer DNA from *Agrobacterium* to tobacco cells.

A T-DNA-free vector that can be maintained in both *E. coli* and *A. tumefaciens* was obtained by removing the entire T-DNA region of the conventional binary vector pCAMBIA1301 (Cambia, AU). This was accomplished by simultaneously ligating a 5.9 kb SacII – SphI fragment of pSIM1301 with 2 fragments amplified from pCAMBIA1301 using the oligonucleotides pairs: 5'-CCGCGGTGATCACAGGCAGCAAC - 3' (SEQ ID NO. 58) and 5'-AAGCTTCCAGCCAGCCAACAGCTCCCCGAC-3' (SEQ ID NO. 59) , and 5'-AAGCTTGGCTACTAGTGCGAGATCTCTAAGAGAAAAGAGCGTTTA-3' (SEQ ID NO. 60), and 5'-GCATGCTCGAGATAGGTGACCACATACAAATGGACGAACGG-3' (SEQ ID NO. 61), respectively.

To make it possible to screen against backbone integration events, an expression cassette comprising the *Agrobacterium* isopentenyl transferase (*IPT*) gene driven by the Ubi3 promoter and followed by the Ubi3 terminator (SEQ ID NO.: 3) was inserted as 2.6 kbp SacII fragment into the backbone of the T-DNA-free vector described above, yielding pSIM100-OD-IPT. Transformed plant cells expressing the *IPT* gene are expected to accumulate cytokinins and grow into abnormal shoots that cannot develop roots.

The 0.4 kb P-DNA fragment described in Example 1 was inserted into pSIM100-OD-IPT to generate pSIM111 (Figure 1; SEQ ID NO.: 4).

To test whether pSIM111 can be used to obtain transformed plants carrying P-DNAs (including any sequences located between P-DNA termini) without the additional vector backbone, a neomycin phosphotransferase (*NPTII*) gene expression cassette was inserted into the P-DNA of pSIM111 to create pSIM108 (Figure 1).

The efficacy of P-DNA termini in supporting DNA transfer was tested by comparing transformation frequencies between pSIM108 and a control vector that contained a modified P-DNA with conventional T-DNA borders. This control vector, designated pSIM109, was generated by amplification of the entire P-DNA containing the *NPTII* gene expression cassette with the oligonucleotide pairs: 5'-

ACTAGTGTTTACCCGCCAATATATCCTGTCAGAG-3' (SEQ ID NO. 62), and 5'-AAGCTTTGGCAGGATATATTGTGGTGTAAACGAAG-3' (SEQ ID NO. 63). A second control vector that was used for these experiments is the conventional binary vector pBI121 (Genbank accession number AF485783), which contains the same *NPTII* expression cassette inserted on a regular T-DNA. The binary vectors were introduced into *Agrobacterium tumefaciens* LBA4404 cells as follows. Competent LB4404 cells (50 uL) were incubated for 5 minutes at 37°C in the presence of 1 μg of vector DNA, frozen for about 15 seconds in liquid nitrogen (about –196°C), and incubated again at 37°C for 5 minutes. After adding 1 mL of liquid broth (LB), the treated cells were grown for 3 hours at 28°C and plated on LB/agar containing streptomycin (100 mg/L) and kanamycin (100 mg/L). The vector DNAs were then isolated from overnight cultures of individual LBA4404 colonies and examined by restriction analysis to confirm the presence of intact plasmid DNA.

Test transformations of the model plant tobacco were carried out by growing a 10-fold dilution of overnight-grown LBA4404::pSIM108 cells for 5-6 hours, precipitating the cells for 15 minutes at 2,800 RPM, washing them with MS liquid medium (Phytotechnology) supplemented with sucrose (3%, pH 5.7) and resuspending the cells in the same medium to an OD_{600nm} of 0.2. The suspension was then used to infect leaf explants of 4-week-old *in vitro* grown *Nicotiana tabacum* plants. Infected tobacco explants were incubated for 2 days on co-culture medium (1/10 MS salts, 3% sucrose, pH 5.7) containing 6 g/L agar at 25°C in a Percival growth chamber (16 hrs light) and subsequently transferred to M401/agar medium containing timentine (150 mg/L) and kanamycin (100 mg/L). The number of calli per explant that developed within the next 4 weeks is shown in Table 3. Our data demonstrate that P-DNAs delineated by either native termini or conventional T-DNA borders are about 50% more effective in transforming tobacco than T-DNAs. The increased efficiency of P-DNA transfer may be due to either its different CG content or other unknown structural features of the P-DNA.

Example 3

Potato transformation with P-DNA vectors

This Example demonstrates that a P-DNA can be used in a similar way as a T-DNA to transfer DNA from *Agrobacterium* to potato cells.

Potato transformations were carried out by infecting stem explants of 4-week-old *in vitro* grown Russet Ranger plantlets with *Agrobacterium* strains according to the following procedure. Ten-fold dilutions of overnight-grown cultures were grown for 5-6 hours, precipitated for 15 minutes at 2,800 RPM, washed with MS liquid medium (Phytotechnology) supplemented with sucrose (3%, pH 5.7), and resuspended in the same medium to an OD_{600nm} of 0.2. The resuspended cells were then used to infect 0.4-0.6 mm internodal potato segments. Infected stems were incubated for 2 days on co-culture medium (1/10 MS salts, 3% sucrose, pH 5.7) containing 6 g/L agar at 22^{O}C in a Percival growth chamber (16 hrs light) and subsequently transferred to callus induction medium (CIM, MS medium supplemented with 3% sucrose 3, 2.5 mg/L of zeatin riboside, 0.1 mg/L of naphthalene acetic acid, and 6g/L of agar) containing timentine (150 mg/L) and kanamycin (100 mg/L). After 1 month of culture on CIM, explants were transferred to shoot induction medium (SIM, MS medium supplemented with 3% sucrose, 2.5 mg/L of zeatin riboside,0.3 mg/L of giberelic acid GA3, and 6g/L of agar) containing timentine and kanamycin (150 and 100 mg/L respectively). After 3-4 weeks, the number of explants developing transgenic calli and/or shooting was counted. As shown in tobaco, the number of stem explants infected with pSIM108 that showed calli was higher than those in control experiments with the conventional binary vector pBI121 (Table 3). Shoots that subsequently arose from these calli could be grouped into two different classes. The first class of shoots was phenotypically indistinguishable from control shoots transformed with LBA::pBI121. The second class of shoots displayed an IPT phenotype. Shoots of the latter class were stunted in growth, contained only very small leaves, displayed a light-green to yellow color, and were unable to root upon transfer to hormone-free media. To confirm that shoots with an IPT phenotype contained the *IPT* gene stably

integrated in their genomes, all shoots were transferred to Magenta boxes containing MS medium supplemented with 3% sucrose and timentine 150 mg/L, allowed to grow for 3 to 4 additional weeks, and used to isolate DNA. This plant DNA served as template in PCR reactions with an oligonucleotide pair designed to anneal to the IPT gene: 5'- GTC CAA CTT GCA CAG GAA AGA C-3', and 5'- CAT GGA TGA AAT ACT CCT GAG C-3'. As shown in Table 4, the PCR experiment confirmed a strict correlation between IPT phenotype and presence of the *IPT* gene. The presence of backbone DNA was also examined in plants obtained from a transformation with pBI121. This was done by performing PCR reactions on DNA isolated from the transformation events with the 'pBI121 backbone primers': 5'-CGGTGTAAGTGAACTGCAGTTGCCATG-3' (SEQ ID NO. 64), and 5'-CATCGGCCTCACTCATGAGCAGATTG-3' (SEQ ID NO. 65). Amplification of a 0.7 kbp band is indicative for backbone integration. By comparing the data presented in Table 4, it can be concluded that backbone integration frequencies are similar for P-DNA vectors and T-DNA vectors.

A second PCR experiment was carried out to test whether *IPT* -free plants did not contain any other backbone sequences. Because the *IPT* expression cassette is positioned close to the left border-like sequences, the oligonucleotide pair for this experiment was designed to anneal to backbone sequences close to the right border-like sequence: 5'- CACGCTAAGTGCCGGCCGTCCGAG-3' (SEQ ID NO. 66), and 5'-TCCTAATCGACGGCGCACCGGCTG-3' (SEQ ID NO. 67). Data from this experiment confirm that plants that are positive for the *IPT* gene are also positive for this other part of the backbone.

Similar experiments were carried out with the potato variety Russet Burbank. Based on an assessment of IPT phenotypes, the backbone integration frequencies for pSIM108 and pSIM109 were shown to be comparable to those in Russet Ranger (see Tables 4 and 5).

Example 4

Potato invertase inhibitor gene

Using conventional transformation methods, this Example demonstrates that overexpressing a novel potato invertase inhibitor gene enhances the processing and health characteristics of potato tubers.

The following primers were designed to amplify a new potato homolog of the tobacco vacuolar invertase inhibitor Nt-inhh1 (Greiner et al., Nature Biotechnology, 17, 708-711, 1999): 5'- AAAGTTGAATTCAAATGAGAAATTTATTC–3' (SEQ ID NO. 68), and 5'- TTTTAAGCTTTCATAATAACATTCTAAT –3' (SEQ ID NO. 69). The amplification reaction was performed by mixing the following components: 4 µl plant DNA, 2 µl forward primer (10 pM/ml), 2 µl reverse primer, 25 µl Hot Start Master Mix (Qiagen Catalog Nr. 203443), and 17 µl water. This reaction mix was subjected to the following polymerase chain reaction (PCR) conditions using a PTC-100 thermocycler (MJ Research): (1) 5 minutes at 95^{O}C (1 cycle), (2) 1 minute at 94^{O}C, 1 minute at 45^{O}C and 4 minutes at 72^{O}C (35 cycles), and (3) 10 minutes at 72OC (1 cycle). The total product was loaded on a 0.8% agarose gel, and a 540 base pair band was purified from gel using QIAquick Gel Extraction Kit (Qiagen, CA). This purified fragment was then ligated into pGEM-T Easy (Promega, WI) and transformed into *E. coli* DH5-alpha using Max Efficiency Competent Cells (GibcoBRL, MD). Sequence analysis of recombinant plasmid DNA isolated from transformed DH5-alpha revealed the presence of a single open reading frame consisting of 543 base pairs that encodes for a putative 181-amino acid protein (SEQ ID NO.: 5); clustal-aligment revealed 70% homology to Nt-inhh (Figure 2). This high level of homology extends to the 15-amino acid N-terminal domain, indicating that the potato homolog is targeted to the vacuole. Interestingly, the potato invertase inhibitor homolog, designated St-inh1, shares only 43% homology with the patented tobacco cell wall invertase inhibitor designated Nt-inh1 (Patent WO98/04722; Figure 2).

Although the St-inh1 gene is present in unmodified potato tubers, its expression level is inadequate for full inhibition of invertase and reduced cold-induced sweetening. To increase the storage characteristics of potato, the St-inh1 gene was fused to a new tuber-enhanced promoter of the granule-bound starch synthase (GBSS) gene, which is known to promote high levels of gene expression in tubers. The GBSS promoter was isolated from the potato cultivar Russet Ranger by carrying out a PCR reaction using the forward primer 5'-GAACCATGCATCTCAATC-3' (SEQ ID NO. 70) and the reverse primer 5'-GTCAGGATCCCTACCAAGCTACAGATGAAC-3' (SEQ ID NO. 71). Sequence analysis of the amplified product cloned in pGEM-T demonstrated that this new promoter contains 658 basepairs (SEQ ID NO.: 6). The resulting promoter/gene fusion was then ligated to the 3' regulatory sequence of the potato ubiquitin gene (UbiT; SEQ ID NO.: 7), thus ensuring appropriate termination of transcription of the invertase inhibitor gene.

This expression cassette was inserted between T-DNA borders of a binary vector, and the resulting vector pSIM320 was used to transform Russet Ranger as described above. Three cuttings of nine independent transgenic lines were planted in soil and grown for four weeks in a growth chamber (11 hrs light; 20^{O}C). At least 3 minitubers were then harvested from each line and transferred to a refrigerator set at 4^{O}C to induce cold-sweetening. After 4 weeks, the glucose levels in these cold-stored minitubers were determined by using either an Accu-Chek meter and test strips (Roche Diagnostics, IN) or a glucose oxidase/peroxidase reagent (Megazyme, Ireland). These levels were compared with the average glucose levels in both 6 untransformed lines and 6 "vector control" lines transformed with a pSIM110-derived vector lacking the invertase inhibitor gene. As shown in Table 6, three transgenic lines accumulated less than 40% of the glucose in "vector control" lines demonstrating that the potato invertase inhibitor homolog is functionally active.

The following experiment showed that the amount of reducing sugars present in tubers correlates with acrylamide production during tuber processing. Russet Ranger potato tubers were freshly harvested from the field and stored at 4^{O}C to induce cold-sweetening; control tubers were stored at 18^{O}C. After 4 weeks, glucose levels were

determined in both groups of tubers. Subsequently, tubers were washed, blanched for either 8 minutes or 12 minutes at 165°F, cut into 0.290 x 0.290 shoestring strips, dipped in a 1% sodium acid pyrophosphate solution at 160°F, dried at 160°F until 14 ± 2% dryer weight loss is achieved, fried at 390°F for 40 seconds to attain 64 ± 2% first fry moisture, and frozen for 20 minutes at -15°F, shaking the tray 2-3 times in the first 6 minutes. The resulting French fries were then analyzed for acrylamide levels by Covance laboratory (WI). As shown in Table 7, the glucose levels in tubers stored at 18°C were below the detection level of 0.1 mg/g whereas cold-stored tubers contained on average 3.4 mg/g glucose. This table also shows that fries produced from the latter potatoes contain about 10-fold higher levels of acrylamide than fries produced from potatoes stored at 18°C. Even by using a shorter blanch time for 18°C-stored potatoes than for 4°C-stored potatoes to produce fries with a similar color (color ids of 78 and 71, respectively), a 5-fold difference in acrylamide accumulation was obtained (Table 7). Thus, there appears to be a straight correlation between the amount of reducing sugars such as glucose in tubers and the accumulation of acrylamide in fries derived from these tubers.

To determine whether the reduced glucose levels in pSIM320 lines would limit the processing-induced accumulation of acrylamide, cold-stored pSIM320 minitubers were processed by cutting into wedges, blanching for 8 minutes, dipping in 0.5% SAPP for 30 seconds, drying for 4.5 minutes at 160°F, frying for 40 seconds at 380°F, freezing for 15 minutes at –15°F, and finally drying for 3 minutes and 10 seconds at 160°F. The processed material was then shipped to Covance laboratory for acrylamide determinations. As shown in Table 6, French fries obtained from minitubers with the lowest amounts of glucose accumulated the lowest levels of acrylamide. A 40% reduction in glucose levels in lines "320-2" and "320-4" is associated with a 5-fold reduction in acrylamide levels.

Example 5

Leader and trailer sequences associated with the potato *R1* gene

Using conventional transformation methods, this Example demonstrates that a novel leader sequence associated with the potato *R1* gene can be used effectively to enhance the processing and health characteristics of potato tubers. It also predicts that a novel trailer associated with that same gene can be exploited in the same way.

As an alternative to overexpressing the invertase inhibitor gene, methods were developed to limit acrylamide production without using any actual gene sequences. One such method is based on silencing the tuber-expressed *R1* gene. Previously, it was shown that this starch-related gene can be silenced through antisense expression of a 1.9-kb gene fragment derived from that gene (Kossmann et al., *US Patent* 6,207,880). However, the antisense expression of large DNA fragments is undesirable because such fragments contain new open reading frames (Table 1). As a safer approach to the one described above, a small leader sequence associated with the *R1* gene was isolated from potato. This leader was obtained by performing a rapid amplification of cDNA ends with the 5' RACE kit supplied by GIBCO BRL on total RNA from the tubers of Russet Ranger potato plants. Sequence analysis demonstrated that the *R1*-associated leader consists of 179 basepairs (SEQ ID NO.: 8). Both a sense and antisense copy of this leader sequence, separated by the potato Ubiquitin intron (SEQ ID NO.: 9), were placed between the GBSS promoter and UbiT. The resulting expression cassette for the leader sequence associated with R1 is shown in Figure 3 (SEQ ID NO.: 10). A similar cassette containing a spacer derived from the GBSS promoter (SEQ ID NO.: 11) -instead of the Ubi intron- separating the sense and antisense copies of the R1 trailer is shown in (Figure 3; SEQ ID NOs.: 12). Additional variants with a longer version of the GBSS promoter (SEQ ID NO.: 13) are shown in Figure 3 (SEQ ID NOs.: 14-15).

To test the efficacy of the *R1*-associated leader in limiting acrylamide production, the expression cassette shown in Figure 3 was inserted as KpnI – XbaI fragment between T-DNA borders of a binary vector. An *Agrobacterium* LBA4404 strain carrying the resulting vector pSIM332 was used to transform Russet Ranger potato. To induce tuber formation, 25 shoots representing independent transformation events were transferred to soil and placed in a growth chamber (11 hours light, 25^{O}C). After three

weeks, at least 3 minitubers/line were stored for 4 weeks at 4°C to induce starch mobilization. The glucose levels in these cold-stored minitubers were subsequently determined as described in Example 4, and compared with the average glucose levels in untransformed plants and vector controls. As shown in Table 8, minitubers derived from all 25 lines displayed reduced levels of glucose after cold-storage. An approximate 2-fold reduction in acrylamide levels in expected in French fries derived from minitubers displaying reduced *R1* expression levels compared to controls. Much stronger effects of down-regulating *R1* gene expression are anticipated in mature tubers.

As an alternative to the leader-based approach, expression cassettes that contained both a sense and antisense copy of the trailer sequence associated with *R1* were generated. This trailer was obtained by performing a reverse transcription polymerase chain reaction (RT-PCR) on total RNA isolated from microtubers of the potato cultivar Russet Ranger. Complementary DNA was generated using the Omniscript RT Kit (Qiagen, CA) and then used as a template for a PCR reaction with Hot start DNA polymerase (Qiagen, CA) with the gene-specific reverse primer R1-1 (5'-GTTCAGACAAGACCACAGATGTGA-3'). Sequence analysis of the amplified DNA fragment, cloned in pGEM-T demonstrated that the trailer associated with R1 consists of 333 basepairs (SEQ ID NO.: 16). The sense and antisense copies of the trailer were separated by either the Ubi intron or the GBSS spacer- and sandwiched between GBSS promoter and Ubi3 terminator (Figure 3; SEQ ID NOs.: 17-18). Similar versions with the larger GBSS promoter are shown in Figure 3 (SEQ ID NOs.: 19-20).

Glucose and acrylamide levels can be determined as described above. Tubers displaying about 50% or greater reductions in glucose concentrations are expected to also accumulate about 50% less acrylamide during the frying process. The improved health and storage characteristics of modified plants can be confirmed in mature field-grown tubers.

Phosphate levels in potato tubers can be determined by using AOAC Method 995.11 Phosphorus (Total) in Foods (45.1.33 Official Methods of Analysis of AOAC

International, 17th Edition). Samples are prepared by dry ashing in a muffle furnace followed with an acid digestion. The dissolved samples are then neutralized and treated with a molybdate-ascorbic acid solution and compared to a series of phosphorus standards (treated similarly). A dual beam spectrophotometer would be used for the colorimetric analysis at 823 nanometers. A significant decrease in phosphate content, which is beneficial for the environment, is expected.

Example 6

Leader sequence associated with the L-alpha glucan phosphorylase gene

Using conventional transformation methods, this Example demonstrates that a novel leader sequence associated with the potato L-alpha glucan phosphorylase gene can be used to effectively enhance the processing and health characteristics of potato tubers.

Previously, it was shown that cold-induced sweetening can be reduced through antisense expression of 0.9-kb fragments derived from alpha glucan phosphorylase genes (Kawchuk et al., *US Patent* 5,998,701, 1999). However, the antisense expression of these relatively large DNA fragments is undesirable because they contain new and uncharacterized open reading frames that may impact the nutritional quality of foods if expressed in transgenic plants (Table 1).

As a safer approach to the one described above, small leader and trailer sequences that are associated with a L-type glucan phosphorylase gene were isolated from RNA of mature tubers. The primer pair used for this purpose is: 5'-GGATCCGAGTGTGGGTAAGTAATTAAG-3' (SEQ ID NO. 72), and 5'-GAATTCTGTGCTCTCTATGCAAATCTAGC -3' (SEQ ID NO. 73). The resultant leader sequence of 273 bp was amplified and is shown in SEQ ID NO.: 21. Similarly, the "direct" primer, 5'-GGAACATTGAAGCTGTGG–3' (SEQ ID NO. 74), was used with an oligo-dT primer to amplify a 158 bp "trailer sequence" that is associated with the L-type phosphorylase gene (SEQ ID NO.: 22).

Expression cassettes were then designed using these trailer or leader sequences to modify the expression of L-type phosphorylase gene and, in so doing, lowering acrylamide levels in fried products by limiting starch mobilization. These cassettes were constructed in a similar way as described in Example 5, and are depicted in Figure 3 (SEQ ID Nos.: 23-26). An *Agrobacterium* strain containing a binary vector with this expression cassette, designated pSIM216, was used to infect potato stems, and generate 25 transgenic plants. Minitubers derived from these plants were stored for 4 weeks at 4°C to induce cold-sweetening. The cold-stored minitubers were then analyzed for glucose levels. As shown in Table 9, minitubers from all transgenic lines displayed reduced glucose levels.

Four lines that displayed at least 50% reduced glucose concentrations (lines 216-2, 216-5, 216-10, and 216-21) were used to assess processing-induced acrylamide levels. Although acrylamide levels in fried tubers derived from the first three lines were similar to those of controls, French fries that were derived from line 216-21 accumulated only 45% of the wild-type acrylamide levels (136 vs. 305 parts per billion). These results confirm the experiments described in Example 4 for tubers overexpressing the potato invertase inhibitor gene, in that relatively large reductions in glucose (and fructose) concentrations are needed to limit the heating-induced acrylamide accumulation in cold-stored minitubers. Because silencing of the phosphorylase gene is expected to be more effective in mature "216" tubers, reductions in acrylamide levels are also anticipated to be more pronounced in the French fries produced from such tubers. The improved health and storage characteristics of modified plants can be confirmed in mature tubers.

Example 7

Modified polyphenol oxidase gene

Using conventional transformation methods, this Example demonstrates that a modified polyphenol oxidase gene lacking a functional copper-binding site can be used effectively to reduce bruise susceptibility in tubers.

Previously, it was shown that black spot bruise susceptibility can be reduced through antisense expression of the 1.8-kb *PPO* gene (Steffens, *US Patent* 6,160,204, 2000). However, expression of the reverse complement of this large gene is undesirable because it contains new and uncharacterized open reading frames encoding peptides consisting of more than 100 amino acids, which may potentially impact the nutritional quality of foods (Table 1). As a safer approach to the one described above, the *PPO* gene was modified to encode a non-functional protein.

The wild-type potato *PPO* gene was isolated from Russet Ranger by using a polymerase chain reaction (PCR) method. First, genomic DNA was isolated from sprouts of Russet Ranger. The potato *PPO* gene was then amplified from the potato genomic DNA using DNA polymerase and oligonucleotide primers: 5': CGAATTCATGGCAAGCTTGTGCAATAG-3' (PPO-F) (SEQ ID NO. 75), and 5'-CGAATTCTTAACAATCTGCAAGACTGATCG-3' (PPO-R) (SEQ ID NO. 76). These were designed to complement the 5'- and 3'-ends of the potato PPO gene. The amplified 1.6 kb fragment was cloned into a pGEM-T EASY vector (Promega) and confirmed to represent a functional PPO gene by sequence analysis (SEQ ID NO.: 27).

The copper binding domain in potato PPO was identified by aligning this protein with a sweet potato PPO protein that was shown to contain conserved Cysteine (Cys) residue at position 92, Glutamine residue (Glu) at position 236, and Histidine (His) residues at positions 88, 109, 118, 240, 244 and 274 coordinating the two active site coppers (Klabunde et al., Nature Structural Biol., 5: 1084-1090, 1998). These Cys, Glu, and His residues are also present in potato PPO.

The inactive *PPO* gene was created by using a PCR mutation replacement approach. Three fragments were amplified by Proof Start Taq DNA Polymerase (Qiagen) using 3 pairs of primers and wild-type Russet Ranger PPO as a template. The sequences of the first pair, designated P1-F and P2-R, respectively, are: 5'-GAGAGATCTTGATAAGACACAACC -3' (SEQ ID NO. 77), and 5'-CATTACC[1]ATAAGCC[2]CAC[3]TGTATATTAGCTTGTTGC- 3' (SEQ ID NO. 78) (1: "A" to "C" mutation, resulting in Cysteine to Glycine substitution at position 186; 2:

"A" to "C" mutation, resulting in Cysteine to Tryptophan substitution at position 183; 3: "A" to "C" mutation, resulting in Histine to Glutamine substitution at position 182). The sequences of the second pair, designated P3-F and P4-R, respectively, are 5'- GTGCTTATAGAATTGGTGGC -3' (SEQ ID NO. 79), and 5'- TAGTTCCCGGGAGTTCAGTG -3' (SEQ ID NO. 80). The sequences of the third pair, designated P5-F and P6-R, respectively, are 5'-CTCCCGGGAACTATAGG[4]AAACATTCCTCT[5]CGGTCCTGTCCACATCTGGTC -3' (SEQ ID NO. 81) and 5'-GTGTGATATCTGTTCTTTTCC-3' (SEQ ID NO. 82) (4: "A" to "G" mutation, resulting in Glutamine to Glycine substitution at position 326; 5: "A" to "T" mutation, resulting in Histine to Leucine substitution at position 330).

An 80 bp fragment was amplified using primer P1-F and P2-R and digested with BglII. This fragment contains one sticky end (BglII) and one blunt end, and carries three mutations in copper binding site I. A 0.4 kb fragment amplified using primer P3-F and P4-R and digested with XmaI contains one blunt end and one sticky end (XmaI). A 0.2 Kb fragment was amplified using primer P5-F and P6-R and digested with XmaI and EcoRV. This third fragment with a sticky end (XmaI) and a blunt end (EcoRV) has two mutations in copper binding site II. The BglII and EcoRV fragment from cloned wild-type potato PPO was then replaced with the above three ligated PCR amplified fragments. The presence of a total of 5 point mutations in the modified *PPO* gene was confirmed by sequence analysis (SEQ ID NO.: 28). To create an expression cassette for modified *PPO* (*mPPO*), the following four fragments were simultaneously ligated together: (1) a BamHI-HindIII fragment containing the GBSS promoter, (2) a HindIII-SacI fragment containing mutant PPO, (3) a SacI-KpnI fragment containing the Ubi-3 terminator, and (4) plasmid pBluescript, digested with KpnI and BamHI. This expression cassette was then inserted between borders of a binary vector to create pSIM314.

The efficacy of the *mPPO* gene expression cassette was assessed by transforming Russet Ranger stem explants with pSIM314. Nodal cuttings of transgenic plants containing this expression cassette were placed on MS medium supplemented with

7% sucrose. After a 5-week incubation period in the dark at 18°C, microtubers were isolated and assayed for PPO activity. For this purpose, 1 g of potato tubers was pulverized in liquid nitrogen. This powder was then added to 5 ml of 50 mM MOPS (3-(N-morpholino) propane-sulfonic acid) buffer (pH 6.5) containing 50 mM catechol, and incubated at room temperature with rotation for about 1 hour. The solid fraction was then precipitated, and the supernatant transferred to another tube to determine PPO activity by measuring the change of OD-410 over time. As shown in Table 10, microtubers isolated from some of the transgenic lines displayed a significantly reduced polyphenol oxidase activity compared to either untransformed controls or controls transformed with a construct not containing the mutant *PPO* gene. The strongest reduction in PPO activity was observed in lines "314-9", "314-17", and "314-29". To test whether expression of the mutant PPO gene also reduced PPO activity in minitubers, rooted plantlets of transgenic lines were planted in soil and incubated in a growth chamber for 4 weeks. A PPO assay on isolated minitubers demonstrated that reduced PPO activity in microtubers correlated in most cases with reduced activities in minitubers (Table 10). Transgenic lines displaying a reduced PPO activity can be propagated and tested both in the greenhouse and the field to confirm the "low bruise" phenotype in mature tubers. Because micro- and minitubers express a variety of polyphenol oxidases, some of which share only limited sequence homology with the targeted polyphenol oxidase that is predominantly expressed in mature tubers, an even more profound reduction of PPO activity may be anticipated in the mature tubers of lines such as "314-9" and "314-17". The data indicate that overexpression of a functionally inactive PPO gene can result in reduced bruise susceptibility. The improved health and storage characteristics of modified plants can also be confirmed in mature field-grown tubers.

Example 8

Trailer sequence of a polyphenol oxidase gene that is specific for the non-epidermal tissues of potato tubers

Using conventional transformation methods, this Example demonstrates that a novel trailer sequence associated with the potato *PPO* gene can be used effectively to reduce bruise susceptibility in tubers.

Reverse transcription PCR was used to also isolate the trailer sequence associated with the PPO gene expressed in potato tubers. The primers for the first PCR reaction were PPO-1 (5'-GAATGAGCTTGACAAGGCGGAG-3', (SEQ ID NO. 83)) and oligo-dT; primers for a second nested PCR reaction were PPO-2 (5'-CTGGCGATAACGGAACTGTTG-3', (SEQ ID NO. 84)) and oligo-dT. Sequence analysis of the amplified DNA fragments cloned into pGEM-T revealed the presence of a 154-bp trailer (SEQ ID NO.: 29). A sense and antisense copy of this trailer, separated by the Ubi intron, was then fused to the GBSS promoter and Ubi3 terminator as described above to generate an expression cassette shown in Figure 3 (SEQ ID NO.: 30). An alternative construct containing the trailer segments separated by a GBSS spacer is shown in Figure 3 (SEQ ID NO.: 31). Similar versions with the larger GBSS promoter are shown in Figure 3 (SEQ ID NOs.: 32-33). Interestingly, the trailer of the *PPO* gene that is predominantly expressed in mature tubers (indicated with P-PPO3 in Figure 4) is different from the trailer of *PPO* genes that are predominantly expressed in other tissues including microtubers (indicated with PPOM-41 and PPOM-44 in Figure 4). Because of the low homology between trailers associated with different *PPO* genes, the use of the P-PPO3 trailer will result in a silencing of the mature tuber-specific *PPO* gene only. This very specific gene silencing would be difficult to accomplish with sequences derived from the *PPO* gene itself, thus demonstrating the advantage of using non-coding sequences for gene silencing. To visualize the extend of PPO activity, 0.5 mL of 50 mM catechol was pipetted on the cut surfaces of sliced genetically modified minitubers. Compared to controls, visual browning of the tuber regions was about 5 to 10-fold reduced. Interestingly, though, no reduced browning was observed in the potato skin. It

appears that the trailer sequence used specifically silenced the *PPO* gene that is predominantly expressed in cortex and pith but not in the epidermal skin. This unexpected finding may be beneficial for tubers to protect themselves against some pathogens attempting to infect through the skin because the *PPO* gene may play some role in certain defense responses. To quantitatively determine PPO activity, an assay was performed as described in Example 7. Table 11 shows up to 80% reduction of PPO activity in transformed minitubers compared to untransformed controls. The level of reduction is expected to be even greater in mature tubers because these tubers express the targeted *PPO* gene more predominantly than mini- and microtubers. The improved characteristics of lines such as "217-7" and "217-26" can be confirmed in mature tubers.

Example 9

An expression cassette to increase levels of resistant starch

Increasing the amylose/amylopectin ratios in tubers can further enhance the nutritional value of potato products. One method that makes it possible to increase amylose content is based on the antisense expression of genes encoding for the starch branching enzyme (*SBE*) I and II (Schwall et al., *Nature Biotechnology* 18: 551-554, 2000). The disadvantages of this method are that (1) the efficiency of simultaneously silencing two different genes through exploitation of antisense technologies is very low, (2) the antisense expression of the relatively large *SBE-I* and *SBE-II* gene sequences results in the undesirable expression of open reading frames (Table 1) (3) corresponding constructs that harbor the two antisense expression cassettes are unnecessarily large and complex, thus, increasing chances of recombination and lowering transformation frequencies.

Our approach to increase amylose content in potato is based on the expression of the trailer sequences that are associated with both genes. These trailers (SEQ ID No.:34 and 35) were isolated with the primer pairs 5'-GTCCATGATGTCTTCAGGGTGGTA –3' (SEQ ID NO. 85), and 5'-CTAATATTTGATATATGTGATTGT –3' (SEQ ID NO. 86), and 5'-

ACGAACTTGTGATCGCGTTGAAAG –3' (SEQ ID NO. 87), and 5'-ACTAAGCAAAACCTGCTGAAGCCC –3' (SEQ ID NO. 88). A single promoter drives expression of a sense and antisense fusion of both trailers, separated by the Ubiquitin-7 intron, and followed by the Ubiquitin-3 terminator. The size of the entire expression cassette is only 2.5-kb.

Example 10

Development of marker-free transformation methods

This Example demonstrates that plants can be transformed effectively without to need for stable integration of selectable marker genes.

This method is the first to take advantage of the phenomenon that DNAs targeted to the nuclei of plant cells often fails to subsequently integrate into the plant cell's genome. The inventors made the surprising discovery that it is possible to select for cells that temporarily express a non-integrating T-DNA containing a selectable marker gene by placing infected explants for 5 days on a plant medium with the appropriate selective agent. A second phenomenon that was applied to develop the current method is that T-DNAs from different binary vectors often target the same plant cell nucleus. By using two different binary vectors, one containing the selectable marker on a T-DNA, and the other one carrying a T-DNA or P-DNA with the actual sequences of interest, it was possible to apply a transient selection system and obtain populations of calli, shoots or plants, a significant portion of which represents marker-free transformation events.

A conventional binary vector designated pSIM011 was used to represent the vector with the "sequence of interest", which is, in this test case, an expression cassette for the beta glucuronidase (*GUS*) gene located on a conventional T-DNA. The second binary vector that was used for these experiments contains an expression cassette comprising the neomycin phosphotransferase (*NPTII*) gene driven by the strong promoter of the Ubiquitin-7 gene and followed by the terminator sequences of the nopaline synthase (nos) gene between the borders of the T–DNA of a pSIM011-derivative.

Surprisingly, a strong level of transient *NPTII* gene expression levels could also be obtained by replacing the nos terminator with the terminator of the yeast alcohol dehydrogenase 1 (ADH1) gene (Genbank accession number V01292, SEQ ID NO. 56). This finding is interesting because the yeast ADH1 terminator does not share homology with any plant terminator. Importantly, it should be noted here that many yeast terminators do not function adequately in plants. For instance, almost no *GUS* gene expression was observed in a similar experiment as described above with the *GUS* gene followed by the yeast iso-1-cytochrome c (CYC1) terminator (Genbank accession number SCCYT1). An improved vector carrying the selectable marker gene *NPTII* was generated by replacing the nos terminator with the yeast ADH1 terminator. The binary vector containing a selectable marker gene for transient transformation is designated "LifeSupport" (Figure 5).

Potato stem explants were simultaneously infected with two *A. tumefaciens* LBA4404 strains containing pSIM011 and LifeSupport, respectively. A 1/10 dilution of overnight-grown cultures of each strain were grown for 5-6 hours before they were precipitated, washed and resuspended an OD_{600nm} of 0.4 as described in Example 3. The resuspended cells were then used to infect 0.4-0.6 cm internodal potato segments at a final density of each bacteria of 0.2 (OD_{600nm}). Infected stems were treated as in Example 3 with a main difference: the selection with kanamycin was limited to the first 5 days of culture on callus induction medium. Then, explants were allowed to further develop in fresh CIM and SIM containing only timentine 150 mg/L but no selective antibiotic. Within about 3 months from the infection day leaves from shoots derived from calli developed in 40-60% of the infected stems were both tested for *GUS* expression and PCR analyzed to identify events that contained the sequences of interest but no marker gene. As shown in Table 12, 11% of shoots represented marker-free transformation events.

The two-strain approach described above was also used to transform tobacco. Shoots that developed within about 2 months were GUS assayed and PCR analyzed. The high frequency of marker-free transformation events identified (18%) implies that the developed method is applicable to plant species other than potato (Table 12).

Importantly, sequential rather than simultaneous infection with the two different *Agrobacterium* strains resulted in an increase in the efficiency of marker-free transformation. The surprising effect of sequential infections was discovered by infecting potato stem explants with the *Agrobacterium* strain containing pSIM011, placing the infected explants on co-cultivation plates for 4 hours, and then re-infecting them with the LifeSupport vector. The doubly infected explants were treated as previously described in this example. As shown in Table 13, the lag time of 4 hours between the two different infections resulted in a 2-fold increased frequency of marker-free transformation events in potato.

Example 11

Precise breeding with pSIM340

This Example demonstrates the efficacy of precise breeding. The health and agronomic characteristics of potato plants are enhanced by inserting potato genetic elements (see Examples 1, 4, and 7) into potato, using marker-free transformation (Example 10).

A binary vector containing two expression cassettes for the invertase inhibitor and mutant polyphenol oxidase genes inserted between P-DNA termini, designated pSIM340 (Figure 1), was created by inserting both expression cassettes of mutant *PPO* and invertase inhibitor into a binary vector pSIM112'. Potato stem explants were infected simultaneously with pSIM340 and a further improved LifeSupport vector. The infected explants were then co-cultivated, subjected to transient selection, and induced to proliferate and develop shoots as discussed earlier. After 3 months, small shoots were transferred to new media and allowed to grow for 3 additional weeks. Shoots were then phenotypically analyzed, and leaf material was collected for molecular analyses to determine the presence of backbone, marker gene and P-DNA with the sequences of interest, as described in Examples 2 and 3. As shown in Table 14, 1.2% of events represented a plant that contained the modified P-DNA of pSIM340 without LifeSupport. This frequency of maker-free transformation is lower

than found for a T-DNA, again revealing a functional difference between P-DNA and T-DNA.

Example 12

Selecting against stable integration of LifeSupport T-DNAs

This Example demonstrates that the efficiency of precise breeding methods can be increased by selecting against stable integration of LifeSupport T-DNAs using the bacterial cytosine deaminase gene.

The previous example demonstrates that the efficiency of marker-free transformation is several-fold lower with a modified P-DNA than with a conventional T-DNA. To improve the efficiency of generating shoots only containing a modified P-DNA, an expression cassette for a suicide gene fusion comprising the bacterial cytosine deaminase (*codA*) and uracil phosphoribosyltransferase (*upp*) genes (InvivoGen, CA) was inserted between T-DNA borders of the LifeSupport vector, generating pSIM346 (Figure 5). Potato stem explants were infected with one strain carrying pSIM340 and the other carrying pSIM346, and subsequently placed on the following media: (1) co-cultivation media for 2 days, (2) CIMTK media to select for transient marker gene expression for 5 days, (3) CIMT media to allow proliferation of plant cells that transiently expressed the marker gene for 30 days, (4) SIMT media with 500 mg/L of non-toxic 5-fluorocytosine (5-FC), which will be converted by plant cells expressing *codA::upp* into the toxic toxic 5-fluorouracil (5-FU), to select against stable integration of the LifeSupport TDNA. Callus gave rise to shoots on SIMT within 4 weeks. These shoots were transferred to MS media with timentin and allowed to grow until sufficient tissue was available for PCR analysis. DNA was then extracted from 100 shoots and used to determine the presence of P-DNA, LifeSupport and backbone. As shown in Table 15, none of the shoots analyzed contained a LifeSupport T-DNA, indicating, for the first time, that the *codA::upp* gene fusion can be used as negative selectable marker prior to regeneration. More importantly, our results demonstrate that a negative selection against LifeSupport T-DNA integration increases the frequency of shoots that only contain a modified P-DNA. By coupling a

positive selection for transient marker gene expression with a negative selection against stable integration of the *codA::upp* gene fusion, the frequency of shoots only containing a modified P-DNA is about 5-fold higher than by only employing the positive selection for transient marker gene expression (Table 15).

An even greater increase in the efficiency of marker-free transformation was obtained by using the LifeSupport vector pSIM350 (Figure 5), which is similar to pSIM346 but contains the *codA* gene instead of the *codA::upp* gene fusion. Potato stem explants simultaneously infected with pSIM340 and pSIM350 were treated as described above, and 51 resulting shoots were molecularly tested for the occurrence of events only containing the T-DNA region from pSIM340. Interestingly, this PCR analysis revealed that some shoots contained the *codA* gene (Table 15). This finding demonstrates that *codA* is not as tight a negative selectable marker as *codA::upp* in plants. More importantly, a large number of shoots (29%) were shown to represent marker-free transformation events.

Efficiencies can be further increased by not infecting explants simultaneously with pSIM340 and pSIM350 but sequentially. By infecting the explants with pSIM340 and re-infecting them with pSIM350 after 4 hours, marker-free transformation frequencies are expected to be approximately 30-40%.

Example 13

Impairing integration of LifeSupport T-DNAs

This Example demonstrates that the efficiency of precise breeding methods can be increased by impairing integration of the LifeSupport T-DNA into the plant genome using an omega-mutated *virD2* gene.

It has been shown that the omega domain of the *Agrobacterium* protein virD2 is important for the ability of that protein to support T-DNA integration into plant genomes (Mysore et al., *Mol Plant Microbe Interact* 11: 668-83, 1998). Based on this observation, modified LifeSupport vectors were created that contain an expression cassette for an omega-mutated virD2 protein inserted into the SacII site in their

backbone sequences. The expression cassette was obtained by amplifying a 2.2-kb DNA fragment from plasmid pCS45 (courtesy of Dr. Walt Ream –Oregon State University, OR, USA-, SEQ ID NO.: 36). A LifeSupport-derivative carrying this expression cassette, designated pSIM401Ω (Figure 5), was used to support the transformation of potato plants with the modified P-DNA of pSIM340. After transient selection and shoot induction, 100 shoots were molecularly tested for the presence of transgenes. As shown in Table 15, 4.4% of shoots only contained the modified P-DNA, indicating that the use of omega-virD2 increases the efficiency of marker-free transformation about 4-fold (Table 15).

Efficiencies are further improved by increasing the size of the LifeSupport T-DNA from 3.7 kb (in pSIM401Ω) to 8.1 kb (in the pSIM401Ω-derivative designated pSIM341Ω; Figure 5). By regenerating shoots from potato stem explants simultaneously infected with pSIM340 and pSIM341Ω, 7 of 81 analyzed events (7%) were shown to represent marker-free transformation events (Table 15).

A further improvement can be obtained by infecting explants sequentially rather than simultaneously with pSIM340 and LifeSupport. In a similar way as described in Example 10, the frequency of plants that only contain a modified P-DNA can be about doubled by infecting the explants with pSIM340 and re-infecting them with LifeSupport after 4 hours.

Example 14

Development of a 1-strain approach

This Example demonstrates that high frequencies of marker-free transformation can also be obtained by using a single *Agrobacterium* strain that contains both the P-DNA vector and LifeSupport

Two compatible binary vectors were created that can be maintained simultaneously in *Agrobacterium*. Instead of using this system to stably integrate two T-DNAs carrying the DNA-of-interest and a marker gene, respectively (Komari et al. *US Patent* 5731179, 1998), it is intended for integration of only the modified P-DNA.

The first vector, designated pSIM356, contains an expression cassette comprising the *GUS* gene driven by the Ubi7 promoter and followed by UbiT inserted between P-DNA termini. The backbone portion of this vector contains bacterial origins of replication from pVS1 and pBR322, a spectinomycin resistance gene for bacterial selection, and an expression cassette for the *IPT* gene to enable selection against backbone integration in plants (Figure 1). The second vector, designated pSIM363, contains an expression cassette comprising the *NPTII* gene driven by the Ubi7 promoter and followed by the yeast ADH1 terminator inserted between conventional T-DNA borders (Figure 5). The backbone portion of this vector contains bacterial origins of replication from ColE1 (Genbank number V00268) and ori V (Genbank number M20134), and a kanamycin resistance gene for bacterial selection.

The concept of increasing marker-free transformation frequencies using pSIM356 and pSIM363 was tested in 100 tobacco shoots. As shown in Table 16, about 19% of regenerated shoots contained the DNA of interest without marker gene. An increase in marker-free transformation efficiency was also found by applying this 1-strain approach to potato. Nine of 60 independent shoots tested (15%) contained the pSIM340 T-DNA and lacked the LifeSupport T-DNA (Table 16).

The 1-strain approach can be combined with the method described in Example 12 to couple a positive selection for transient marker gene expression with a negative selection against stable integration of the *codA* gene. For this purpose, the LifeSupport vector pSIM365 was developed (Figure 5). An *Agrobacterium* strain carrying this vector together with a P-DNA vector can be used to efficiently develop plants that only contain an expression cassette-of-interest located within a P-DNA stably integrated in their genomes.

Example 15

Precise breeding method relying on a native marker

Apart from transforming crop plants with P-DNAs that only contain the desirable sequences to introduce beneficial traits, the present invention also provides a method

of transforming such plants with P-DNAs that contain an additional native marker gene. Our novel and native marker genes of choice are potato homologs of the *Arabidopsis* vacuolar Na+/H+ antiporter gene and alfalfa *alfin-1* gene. Expression of these genes do not only allow the identification of transformation events, but also provides salt tolerance to transformed plants. High salinity levels in an increasing acreage of agricultural land will therefore less affect potato plants containing the salt tolerance marker.

Two versions of a vacuolar Na+/H+ antiporter homolog, designated *Pst* (Potato salt tolerance) were amplified from cDNA of a late blight resistant variety obtained from the US Potato Genbank (WI), designated "LBR4", using the oligonucleotide pair 5'-CCCGGGATGGCTTCTGTGCTGGCT –3' (SEQ ID NO. 89) and 5'-GGTACCTCATGGACCCTGTTCCGT-3' (SEQ ID NO. 90). Their sequences are shown in SEQ ID NO.:37 and 38. A third gene (SEQ ID NO.:39)with homology to *alfin-1* was amplified from LBR4 potato DNA using the primers 5'-CCCGGGTATGGAAAATTCGGTACCCAGGACTG-3' (SEQ ID NO. 91) and 5'- ACTAGTTAAACTCTAGCTCTCTTGC -3' (SEQ ID NO. 92). The efficacy of the *Pst* genes to function as transformation marker was assessed by inserting a fusion with the Ubi7 promoter between conventional T-DNA borders of a modified pSIM341 vector. After a transient selection period, kanamycin-resistant cells are allowed to proliferate and develop shoots. These shoots are then transferred to media that contain 100-150 mM sodium chloride. Salt-tolerant shoots represent transformation events that contain the T-DNA of the modified pSIM341.

Example 16

Tuber-specific promoter

A newly isolated tuber-specific promoter can replace the GBSS promoter used to develop the expression cassettes described in previous examples. This promoter was isolated from the genome of Russet Burbank potato plants by using the inverse polymerase chain reaction with primers specific for a potato proteinase inhibitor gene (Genbank Accession D17332) (SEQ ID NO. 39). The efficacy of the PIP promoter

was tested by creating a binary vector that contains the GUS gene driven by this promoter and an expression cassette for the NPTII marker gene. A similar construct with the PIP promoter replaced by the GBSS promoter was used as control. Transformed shoots were obtained by infecting stem explants with *Agrobacterium* strains carrying the binary vectors, co-cultivation for 2 days, and selection on CIMTK medium for 2 months. These shoots were transferred to new media to induce root formation, and then planted into soil. Tubers can be assayed for GUS expression after a 3-month growth period in the green house.

Example 17

Preferred constructs and transformation methods for precise breeding

Apart from pSIM340, many other vectors can be used to improve potato plants by transforming them with modified P-DNAs . Two of such vectors contain an expression cassette for a sense and antisense copy of the trailer associated with a *PPO* gene that is expressed in all tuber tissues except for the epidermis (see Example 8). Vector pSIM370 contains an additional expression cassette for a sense and antisense copy of the leader associated with phosphorylase gene (see Example 6). Vector pSIM371 contains a third expression cassette for the potato *alfin-1* homolog (Figure 1).

A third alternative vector, designated pSIM372, contains both an expression cassette for the potato *alfin-1* homolog, and an expression cassette for a sense and antisense copy of a fusion of the *PPO*-associated trailer, *R1*-associated leader, and phosphorylase-associated leader.

The preferred LifeSupport vector for a 1-strain approach is pSIM365. For a 2-strain approach, the preferred vector is pSIM367, which contains expression cassettes for both *NPTII* and *codA* between T-DNA borders, and an additional expression cassette for omega *virD2* in the plasmid backbone (Figure 5).

Potato stem explants are infected with 1 strain carrying both pSIM365 and any of the vectors pSIM370, 371, and 372, or sequentially with 2 strains carrying pSIM366 and

any of the preferred vectors-of-interest, respectively. After a 2-day co-cultivation and a 5-day transient selection period, the explants are transferred to media for proliferation/regeneration and elimination of Agrobacterium. Thirty days later, explants are transferred again to the same media but now also containing 5-FU to eliminate events containing LifeSupport T-DNAs. Shoots that subsequently arise on calli are transferred to regeneration media that may contain 100-200 mM salt to screen for salt tolerant events. The IPT-negative shoots are allowed to root and develop into mature plants. A large proportion of these plants (10%-100%) are predicted to represent marker-free and backbone-free plants containing a P-DNA with nucleotide sequences of interest stably integrated into their genomes.

Table 1. Potentially expressed uncharacterized peptides in antisense potato lines

Gene (size of fragment used)	*Predicted peptides encoded by ORFs in reverse-complemented DNA*
RI (1.9-kb)	MSSTSNVGQD CLAEVTISYQ WVGRVINYNF FLLIHWYTVV EASTGITFQI FPIGIRSEDD RSFYEKADRF AWVT
	MSSESTFSKT PNGRATDVGI PTEEGTFPFR YAILRDLAPT ISLVNSSADI A
	MSEGVGFKSK ILPSFAWRSA NILGSKHVAK QTFPFLARTE TCERTSGMSG VIRATAPSGI SSSPLTDFAT KIVGFS
GLTP (1-kb)	VCSPALKADK SKSADGTCVD HSRRLIVVLV LYPGMGTSYA TAFISSPPIQ YLFPSDPVET FP
	MLGSLVLPKS PENRKQAVPN PHFQEQHLVP EKPHFLDCGQ GFSKLPQMHQ
	MVNFLTQGIV DMETAFGSPK MGGFGKEQFG ACVSRSEMDE SGIGAVMVEQ VCSICSRHFV LSMQI
GHTP (0.9-kb)	MLEGSMWPWN QESMKRAFLN HHFLMLHLFP AQRPPQAADP VCLKHQHMHC GCLSFQLHLS KLAPGDTPLI SSMFALD
	MKLCSSIILS IIKQKQVEIL RACFGFPETK TISVFSSVSW NWHIICKSL
	MTKKPDRKDN IMPYNFPGTK FLQPIFRNFF LPSLCDKLLK KSISVPQAIT PCWKVQCGHG IKKA
PPO (1.8-kb)	TILKLDLHTF NGHFFTASFW NQSHRNSIFI FQSNILQQFS YRQLESNTGN MISITSMNM RQASITPCKL RLIKLICIHS LVHVQKHIEP YIVPIIIRYF IECQYLLLLI FLLCCP
	MKGKEKPREM NLQFFTTNFV STVAISTMNI SLLFKAKRVK GVFIKFPHST RSQLILGYVL LIRRMSRGAD AEFSHRRELV VRNTIDLIGY RRATTVYYIN TFFYMGSTTR LEIRRWYRCS SR
	MEWALARNRI PFFYCPNSLR TSHGKGYDFH RRKRIQSSTN LYLLNPFFSR QLISIHSTSC PHWHGGSKKS DLNRVSRNYP CLHRFFDEVC HRSRCEPEYE GCFQ
SBE A (1.2-kb)	MNNITHSPIL IPFLEQLNPF ISNCHMQPIV KANTPILNGN TKCRHSANIF TNGNCIWEKP MNKIVDQHQI HNSIHISCES KVFLVVPSES HR
	MKFRYPSPPN PIVTSLIILC NAIPRSINDV DGLSRAIKSY ISLSISQNAI VLSPTRA
SBE B (2.6-kb)	MVNIMTSSSM ATKFPSITVQ CNSVLPWQVT SNFIPFVCVL WVEVEYKYQV TTFKHNNLII IIHAAYYLFS
	MAKLVTHEIE VPLSSQGHCE KMDHLVKRNS SINNRRSICQ ARHARIHLFV H
	MFETKLNSGV VWNDWLTVNI RNSNTPNTKL VLLHHVVRTV PSIEIANNFV FLSSRSPFTI DYATIFPVES KF
	MLYTSLYISY LSNSMLLPSW TNLHHSYSLN NLSTYLGLPL PGGNQNQFLP QKQAGQGPAY QKHLRQ

Table 3. Transformation efficiency

Binary vector	Calli/tobacco leaf explant ± SE	Calli/potato stem explant ± SE
pBI121	7.8 ± 0.6	0.31 ± 0.10
pSIM108	10.2 ± 0.6	0.59 ± 0.07
pSIM109	12.8 ± 0.6	0.47 ± 0.05

Table 4. Backbone integration resulting from Russet Ranger transformation

Binary vector	*Total Nr.*	*IPT phenotype*	*PCR$^+$ for IPT*	*PCR$^+$ for 0.6 kb backbone fragment*
pBI121	98	NA	NA	54 (55%)
pSIM108	193	138 (71%)	137 (71%)	NA
pSIM109	133	82 (62%)	80 (60%)	NA

NA: not applicable

Table 5. Backbone integration resulting from Russet Burbank transformation

Binary vector	*Total Nr.*	*IPT phenotype*
PSIM108	79	49 (60%)
PSIM109	72	60 (84%)

Table 6. Acrylamide levels in French fries derived from cold-stored pSIM320 minitubers

Line	*glucose mg/g (%-reduced)*	*acrylamide (PPB)*
Untransformed	10.2	469
Vector control	10.2	NA
320-2	**5.4 (47%)**	**95**
320-4	**5.8 (43%)**	**107**
320-7	8.7 (14%)	353
320-9	**7.4 (27%)**	**137**
320-17	6.0 (41%)	506
320-21	8.5 (16%)	428
320-33	6.6 (35%)	516

NA: not available

Table 7. Acrylamide levels in French fries derived from untransformed mature tubers

	Stored at 18°C (color id.)*	*Stored at 4°C (color id.*)*
Glucose levels	<0.1 mg/g	3.4 mg/g
8-minute blanch	53 PPB (78)	603 PPB (56)
12-minute blanch	28 PPB (84)	244 PPB (71)

*: a higher value indicates a lighter color of the finished Fry product

Table 8. Glucose levels in cold-stored pSIM332 minitubers

Line	*glucose mg/g (%-reduced)*
Untransformed control	11.6 ± 0.5
Vector control	11.5 ± 0.5
332-1	**5.4 (53%)**
332-2	**4.8 (58%)**
332-4	7.0 (39%)
332-5	**5.8 (50%)**
332-6	6.9 (40%)
332-7	6.0 (48%)
332-8	6.8 (41%)
332-9	6.6 (43%)
332-10	**5.4 (53%)**
332-11	6.1 (47%)
332-12	6.4 (44%)
332-13	6.4 (44%)
332-15	7.7 (33%)
332-16	6.5 (43%)
332-17	**5.3 (54%)**
332-18	7.1 (38%)
332-21	6.3 (46%)
332-22	5.4 (53%)
332-23	**4.2 (63%)**
332-31	6.0 (48%)
332-34	6.2 (48%)
332-35	6.4 (44%)
332-39	6.7 (41%)
332-40	7.5 (35%)
332-41	**5.7 (50%)**

Table 9. Glucose levels in cold-stored pSIM216 minitubers

Line	*glucose mg/g (%-reduced)*
Untransformed control	11.6 ± 0.5
Vector control	11.5 ± 0.5
216-2	**5.5 (52%)**
216-3	8.8 (23%)
216-4	7.4 (36%)
216-5	**5.8 (50%)**
216-8	8.4 (27%)
216-10	**5.1 (56%)**
216-11	10.1 (19%)
216-12	9.3 (19%)
216-13	6.4 (44%)
216-15	8.8 (23%)
216-16	9.7 (16%)
216-17	6.4 (44%)
216-19	8.7 (24%)
216-21	**3.2 (72%)**
216-24	9.4 (18%)
216-26	9.3 (19%)
216-29	7.1 (38%)
216-30	8.2 (29%)
216-32	9.3 (19%)
216-34	7.1 (38%)
216-35	7.8 (32%)
216-38	7.1 (38%)
216-42	8.1 (30%)
216-44	9.4 (18%)
216-45	10.2 (11%)

Table 10. PPO activity in potato lines expressing a modified *PPO* gene

Line	*OD-410/gram*	
	micro-tubers (%-reduced)	*mini-tubers (%-reduced)*
Untransformed controls	24.59 ± 2.22	20.07 ± 1.21
Vector controls	22.59 ± 3.36	19.55 ± 1.43
314-1	2.36 (90%)	17.8 (11%)
314-2	41.52 (-76%)	21.3 (-7%)
314-4	18.40 (22%)	**5.4 (73%)**
314-5	8.49 (64%)	19.1 (4%)
314-7	16.04 (32%)	16 (20%)
314-8	14.86 (37%)	17 (15%)
314-9	**5.43 (77%)**	**4.3 (78%)**
314-12	19.35 (18%)	19.6 (2%)
314-13	18.17 (23%)	15.4 (23%)
314-14	18.64 (21%)	17.32 (13%)
314-16	13.92 (41%)	18.2 (9%)
314-17	**5.19 (78%)**	**2.4 (88%)**
314-20	26.66 (-13%)	13.2 (34%)
314-21	11.32 (52%)	17.6 (12%)
314-22	13.45 (43%)	18.8 (6%)
314-23	5.19 (78%)	20.4 (-2%)
314-24	15.10 (36%)	19.6 (2%)
314-25	23.12 (2%)	19 (5%)
314-26	13.45 (43%)	17.8 (11%)
314-27	26.42 (-12%)	19.4 (3%)
314-28	31.85 (-35%)	19.4 (3%)
314-29	3.77 (84%)	14.8 (26%)
314-31	23.83 (-1%)	21.2 (-6%)
314-32	28.78 (-22%)	20 (0%)

Table 11. Table 11. PPO activity in potato minitubers expressing a modified trailer sequence associated with the PPO gene

Line	*OD-410/gram (%-reduced)*
Untransformed controls	20.6 ± 1.3
Vector controls	17.9 ± 2.1
217-1	12.5 (39.4%)
217-4	12.6 (38.6%)
217-5	11.3 (45.0%)
217-6	**6.1 (70.4%)**
217-7	**5.7 (72.5%)**
217-9	10.4 (49.6%)
217-10	15.2 (26.3%)
217-11	15.2 (26.3%)
217-12	**6.6 (67.9%)**
217-14	15.4 (25.4%)
217-15	13.5 (34.6%)
217-16	**6.0 (71.0%)**
217-17	**9.7 (53.0%)**
217-19	**8.6 (58.4%)**
217-21	14.2 (31.1%)
217-22	**9.7 (53.0%)**
217-23	15.2 (26.3%)
217-24	**8.2 (60.1%)**
217-25	11.9 (42.2%)
217-26	**3.1 (84.8%)**
217-27	**6.2 (69.9%)**
217-29	**7.2 (65.1%)**

Table 12. Marker-free transformation with the LifeSupport vector + pSIM011

Plant	*Co-transformed*	*Marker only*	*Gene-of-interest only*	*Untransformed*
Potato	0%	33%	11%	56%
Tobacco	20%	26%	18%	36%

Co-transformed: PCR-positive for both *GUS* and *NPT*
Gene-of-interest only: PCR-positive for *GUS*
Untransformed: Plants are PCR-negative for both *GUS* and *NPT*

Table 13. Sequential potato transformation with the LifeSupport vector and pSIM011

Time window	*Co-transformed*	*Marker only*	*Gene-of-interest only*	*Untransformed*
O hrs	9%	36%	9%	46%
4 hrs	20%	30%	20%	30%

Untransformed: Plants are PCR-negative for marker and gene-of-interest

Table 14. Marker-free transformation with the P-DNA vector pSIM340 + LifeSupport

Plant	*Co-transformed*	*Marker only*	*Gene-of-interest only*	*Untransformed*
Potato	17%	52.8%	1.2%	29%

Co-transformed: PCR-positive for both the *PPO* gene of pSIM340 and the NPT gene from LifeSupport
Untransformed: Plants are PCR-negative for *PPO* and *NPTII*

Table 15. Marker-free potato transformation with pSIM340 + improved LifeSupport vectors

LifeSupport vector	*Co-transformed*	*Marker only*	*Gene-of-interest only*	*Untransformed*
PSIM346	0%	0%	4%	96%
PSIM350	10%	10%	29%	51%
PSIM401Ω	6%	34%	5%	55%
pSIM341Ω	16%	23%	7%	54%

Co-transformed: PCR-positive for both the *PPO* gene of pSIM340 and the NPT gene from LifeSupport
Untransformed: Plants are PCR-negative for *PPO* and *NPTII*

Table 16. Marker-free potato transformation with a single *Agrobacterium* strain carrying both pSIM356 and pSIM363

Plant	*Co-transformed*	*Marker only*	*Gene-of-interest only*	*Untransformed*
Tobacco	50%	15%	19%	16%
Potato	22%	5%	15%	58%

Co-transformed: PCR-positive for both the GUS gene of pSIM356 and the NPT gene from LifeSupport
Untransformed: Plants are PCR-negative for *PPO* and *NPTII*

WHAT IS CLAIMED IS:

1. A method of modifying a trait of a selected plant comprising:

a. stably transforming cells from said selected plant with a desired polynucleotide, wherein said desired polynucleotide consists essentially of a nucleic acid sequence that is native to said selected plant, native to a plant from the same species, or is native to a plant that is sexually interfertile with said selected plant,

b. obtaining a stably transformed plant from said transformed plant cells wherein said transformed plant contains said desired polynucleotide stably integrated into the genome and wherein said desired polynucleotide modifies said trait.

2. The method according to claim 1, further comprising co-transfecting said plant cells with a selectable marker gene that is transiently expressed in said plant cells, and identifying transformed plant cells, and transformed plants obtained from said transformed plant cells, wherein said selectable marker gene is not stably integrated and said desired polynucleotide is stably integrated into the genome.

3. The method of claim 1, wherein the plant is a monocotyledenous plant.

4. The method of claim 3, wherein said monocotyledenous plant is selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

5. The method of claim 1, where in the plant is a dicotyledenous plant.

6. The method of claim 5, wherein said dicotyledenous plant is selected from the group consisting of potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

7. The method of claim 1, wherein said trait is selected from the group consisting of enhanced health and nutritional characteristics, improved storage,

enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased drought tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced cold and frost tolerance, enhanced color, enhanced sweetness, improved vigor, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity.

8. The method of claim 1, wherein said desired polynucleotide comprises a P-DNA, GBSS promoter, Ubi7 promoter, Ubi3 promoter, PIP promoter, modified PPO gene, invertase inhibitor gene, salt tolerance gene, R1-associated leader, phosphorylase-associated leader, R1-associated trailer, SBE-associated trailers, Ubi-intron, GBSS spacer, UbiT.

9. The method of claim 1, wherein said plant cells are transformed via *Agrobacterium*-mediated transformation.

10. The method of claim 9, wherein *Agrobacterium*-mediated transformation relies on the use of at least one binary vector.

11. The method of claim 10, wherein the *Agrobacterium*-mediated transformation method uses a first binary vector and a second binary vector.

12. The method of claim 11, wherein the first binary vector contains said desired polynucleotide and the second binary vector contains a functional selectable marker gene, wherein said functional selectable marker gene is operably linked to a promoter and a terminator.

13. A plant made by the method of claim 1.

14. A method of modifying a trait in a selected plant comprising:

(a) identifying the trait to be modified;

(b) constructing a first polynucleotide consisting essentially of native genetic elements isolated from said selected plant, a plant from the same species, or a

plant that is sexually interfertile with said selected plant, wherein said native genetic elements are capable of modifying the expression of a functional gene that controls said trait

(c) constructing a second polynucleotide comprising a functional selectable marker gene;

(d) co-transfecting plant cells from said selected plant with said first and second polynucleotides;

(e) selecting for the transient expression of said functional selectable marker gene;

(f) screening for plant cells stably transformed with said first polynucleotide but do not contain said second polynucleotide integrated into the genome; and

(g) obtaining a stably transformed plant from said transformed plant cells that exhibit modified expression of said trait.

15. The method of claim 14, wherein in the plant is a monocotyledenous plant.

16 The method of claim 15, wherein said monocotyledenous plant is selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

17. The method of claim 14, where in the plant is a dicotyledenous plant.

18. The method of claim 17, wherein said dicotyledenous plant is selected from the group consisting of avocado, potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

19. The method of claim 14, wherein said trait is selected from the group consisting of enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased

drought tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced cold and frost tolerance, enhanced color, enhanced sweetness, improved vigor, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity.

20. The method of claim 14, wherein said genetic elements comprise at least one of a promoter, sequence of interest, terminator, enhancer, intron, spacer, or regulatory elements.

21. The method of claim 14, wherein said plant cells are transformed via *Agrobacterium*-mediated transformation.

22. The method of claim 21, wherein *Agrobacterium*-mediated transformation relies on the use of at least one binary vector.

23. The method of claim 22, wherein the *Agrobacterium*-mediated transformation method uses a first binary vector and a second binary vector.

24. The method of claim 23, wherein the first binary vector carries said first polynucleotide and the second binary vector carries said second polynucleotide.

25. A method of modifying the expression of a functional gene in a selected plant comprising:

(a) constructing a first polynucleotide consisting essentially of native genetic elements isolated from said selected plant, a plant of the same species as said selected plant, or a plant that is sexually interfertile with said selected plant, wherein said native genetic elements are capable of modifying the expression of said functional gene;

(b) constructing a second polynucleotide comprising a selectable marker gene operably linked to a promoter and terminator;

(c) co-transfecting plant cells from said selected plant with said first and second poylnucleotides;

(d) selecting for the transient expression of said selectable marker gene;

(e) screening for plant cells stably transformed with said first polynucleotide but do not contain said second polynucleotide integrated into the genome; and

(f) obtaining a transformed plant from said transformed plant cells that exhibit modified expression of said gene.

26. The method of claim 25, wherein said plant is a monocotyledenous plant.

27. The method of claim 26, wherein said monocotyledenous plant is selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

28. The method of claim 25, where in the plant is a dicotyledenous plant.

29. The method of claim 28, wherein said dicotyledenous plant is selected from the group consisting of potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

30. The method of claim 25, wherein said plant cells are transfected via *Agrobacterium*-mediated transformation.

31. The method of claim 30, wherein *Agrobacterium*-mediated transformation relies on the use of at least one binary vector.

32. The method of claim 31, wherein the *Agrobacterium*-mediated transformation method uses a first binary vector and a second binary vector.

33. The method of claim 32, wherein the first binary vector carries said first polynucleotide and the second binary vector carries said second polynucleotide.

34. The method of claim 25, wherein said first polynucleotide comprises at least one of a P-DNA, GBSS promoter, Ubi7 promoter, Ubi3 promoter, PIP promoter, modified PPO gene, PPO-associated trailer, invertase inhibitor gene, salt tolerance gene, R1-associated leader, phosphorylase-associated leader, R1-associated trailer, SBE-associated trailers, Ubi-intron, GBSS spacer, UbiT.

35. The method of claim 25, wherein said second polynucleotide comprises at least one of a selectable marker gene, an omega-mutated virD2 polynucleotide, a codA polynucleotide, and a codA::upp fusion polynucleotide.

36. A plant made by the method of claim 25.

37. A transgenic plant exhibiting modified expression of a trait compared to the non-trasgenic plant from which it was derived, wherein said transgenic plant is stably transformed with a desired polynucleotide consisting essentially of native genetic elements isolated from said plant, a plant in the same species, or a plant that is sexually interfertile with said plant, and wherein said polynucleotide modifies the expression of said trait.

38. The plant according to claim 37, wherein said plant is a monocotyledenous plant.

39. The plant according to claim 38, wherein said monocotyledenous plant is selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

40. The plant according to claim 37, wherein said plant is a dicotyledenous plant.

41. The plant according to claim 40, wherein said dicotyledenous plant is selected from the group consisting of potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

42. The trait according to claim 37, wherein said trait is selected from the group consisting of enhanced health and nutritional characteristics, improved storage, enhanced yield, enhanced salt tolerance, enhanced heavy metal tolerance, increased drought tolerance, increased disease tolerance, increased insect tolerance, increased water-stress tolerance, enhanced cold and frost tolerance, enhanced color, enhanced sweetness, improved vigor, improved taste, improved texture, decreased phosphate content, increased germination, increased micronutrient uptake, improved starch composition, improved flower longevity..

43. The desired polynucleotide according to claim 37, wherein said polynucleotide comprises at least one of a P-DNA, GBSS promoter, Ubi7 promoter, Ubi3 promoter, PIP promoter, modified PPO gene, PPO-associated trailer, invertase inhibitor gene, salt tolerance gene, R1-associated leader, phosphorylase-associated leader, R1-associated trailer, SBE-associated trailers, Ubi-intron, GBSS spacer, UbiT.

44. An isolated, border-like nucleotide sequence ranging in size from 20 to 100 bp that shares between 52% and 96% sequence identity with a T-DNA border sequence from *Agrobacterium tumafaciens*.

45. The isolated nucleotide sequence of claim 44 wherin said nucleotide sequence is isolated from a monocotyledenous plant.

46. The isolated nucleotide of claim 45, wherein said monotcotyledenous plant is selected from the group consisting of wheat, turf, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

47. The isolated nucleotide sequence of claim 44, wherein said nucleotide sequence is isolated from a dicotyledenous plant.

48. The isolated nucleotide sequence of claim 47, wherein said dicotyledenous plant is selected from the group consisting of potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

49. The nucleotide sequence of claim 44, isolated from potato, which has a nucleotide sequence shown in either SEQ ID NO. 54 or 55.

50. The nucleotide sequence of claim 44, isolated from wheat, which has a nucleotide sequence shown in either SEQ ID NO. 94 or 95.

51. A method of making a plant stably transformed with a desired polynucleotide comprising:

(a) isolating a P-DNA that is flanked by border-like sequences from said plant wherein said border-like sequences share between 52% and 96% sequence identity with an *Agrobacterium tumafaciens* T-DNA border sequence;

(b) inserting said desired polynucleotide between said P-DNA border-like sequences to form a P-DNA construct; and

(c) transforming a plant cell from said plant with said P-DNA construct; and

(d) recovering a plant from said transformed plant cell stably transformed with said P-DNA construct.

52. The method according to claim 51, wherein said P-DNA construct is carried on a vector comprised of a backbone integration marker gene and transformed plant cells are selected that do not contain said backone integration marker gene.

53. The method according to claim 52, wherein said backbone integration marker gene is selected from the group consisting of is a cytokinin gene, and wherein plant shoots are not selected that exhibit a cytokinin-overproducing phenotype.

54. The method according to claim 53, wherein said backnone integraton marker gene is the IPT gene, and plant shoots are not selected that exhibit an abnormal phenotype or cannot develop roots.

55. The method of claim 52, wherein said plant cells are from a monocotyledenous plant.

56 The method of claim 55, wherein said monocotyledenous plant is selected from the group consisting of wheat, turf grass, cereal, maize, rice, oat, wheat, barley, sorghum, orchid, iris, lily, onion, banana, sugarcane, sorghum, and palm.

57. The method of claim 52, where in the plant cells are from a dicotyledenous plant.

58. The method of claim 57, wherein said dicotyledenous plant is selected from the group consisting of avocado, potato, tobacco, tomato, sugarbeet, broccoli, cassava, sweet potato, pepper, cotton, poinsetta, legumes, alfalfa, soybean, carrot, strawberry, lettuce, oak, maple, walnut, rose, mint, squash, daisy, and cactus.

59. The method of claim 51, wherein said plant cells are transfected via *Agrobacterium*-mediated transformation.

60. The method of claim 59, wherein *Agrobacterium*-mediated transformation relies on the use of at least one binary vector.

61. The method of claim 60, wherein the *Agrobacterium*-mediated transformation method uses a first binary vector and a second binary vector.

62. The method of claim 61, wherein the first binary vector carries said P-DNA construct.

63. The method of claim 61, wherein the second binary vector comprises at least one of a negative selectable marker gene and an omega-mutated virD2 gene, wherein the negative selectable marker gene is positioned within the right T-DNA border and the left T-DNA border, and wherein the omega-mutated virD2 gene is positioned within the backbone of the second binary vector.

64. A vector comprising the nucleotide sequence of claim 44.

65. A P-DNA consisting essentially of, in the 5'- to 3'- direction, a first T-DNA border-like sequence, a promoter, a desired polynucleotide sequence operably linked to the promoter, a terminator, and a second T-DNA border-like sequence, wherein the

border-like sequences have less than 100% sequence identity with T-DNA border sequences.

66. The P-DNA of claim 65, wherein said T-DNA border-like sequences, said promoter, said desired polynucleotide, and said terminator, are all isolated from the same plant, the same plant species, or plants that are sexually interfertile.

67. The P-DNA of claim 65, further consisting essentially of a selectable marker gene.

68. The P-DNA of claim 67, wherein said T-DNA border-like sequences, said promoter, said desired polynucleotide, said terminator and said selectable marker gene, are all isolated from the same plant, the same plant species, or plants that are sexually interfertile.

69. The P-DNA of claim 65, wherein the desired polynucleotide sequence is a sequence upstream or downstream of the coding region of a gene, wherein the upstream sequence is a leader sequence, and wherein the downstream sequence is a trailer sequence.

70. The P-DNA of claim 69, wherein said T-DNA border-like sequences, said promoter, said leader sequence, said trailer sequence, said terminator and said selectable marker gene are all isolated from the same plant, the same plant species, or plants that are sexually interfertile.

71. An isolated nucleotide sequence comprising the GBSS promoter isolated from *S. tuberosum*.

72. The isolated nucleotide sequence of claim 71, which has the nucleotide sequence that is SEQ ID. NO. 6 or 13.

73. A vector comprising the P-DNA of claim 65.

74. A vector according to claim 74, wherein the promoter is a regulatable promoter.

75. A vector according to claim 74, wherein the regulatable promoter is sensitive to temperature.

76. A vector according to claim 75, wherein the regulatable promoter is a wheat wcs120 promoter.

77. A vector according to claim 74, wherein the promoter is under temporal regulation.

78. A vector according to claim 77, wherein the promoter is a carboxylase promoter.

79. A vector according to claim 78, wherein the carboxylase promoter is a maize carboxylase promoter.

80. A vector according to claim 74, wherein the promoter is regulated by any one of abscisic acid, wounding, methyl jasmonate or gibberellic acid.

81. A vector according to claim 80, wherein the promoter is a promoter selected from either a Rab 16A gene promoter, an α-amylase gene promoter or a pin2 gene promoter.

82. A vector according to claim 73, wherein the promoter is a tissue-specific promoter.

83. A vector according to claim 69, wherein the leader sequence is a part of a 5'-untranslated region associated with a gene that is endogenous to a cell of the selected plant species.

84. A vector according to claim 69, wherein the 5'- untranslated region is upstream of a start codon of a gene that is selected from the group consisting of a PPO gene, an R1 gene, a HOS1 gene, a S-adenosylhomocysteine hydrolase gene, a class II cinnamate 4-hydroxylase gene, a cinnamoyl-coenzyme A reductase gene, a cinnamoyl

alcohol dehydrogenase gene, a caffeoyl coenzyme A O-methyltransferase gene, an actin depolymerizing factor gene, a Nin88 gene, a Lol p 5 gene, an allergen gene, a P450 hydroxylase gene, an ADP-glucose pyrophosphorylase gene, a proline dehydrogenase gene, an endo- 1,4-beta-glucanase gene, a zeaxanthin epoxidase gene, and a 1-aminocyclopropane-1-carboxylate synthase gene.

85. A vector according to claim 69, wherein the trailer sequence is a part of the 3'-untranslated region associated with a gene that is downstream of a termination codon of a gene selected from the group consisting of a PPO gene, an R1 gene, a HOS1 gene, a S-adenosylhomocysteine hydrolase gene, a class II cinnamate 4-hydroxylase gene, a cinnamoyl-coenzyme A reductase gene, a cinnamoyl alcohol dehydrogenase gene, a caffeoyl coenzyme A O-methyltransferase gene, an actin depolymerizing factor gene, a Nin88 gene, a Lol p 5 gene, an allergen gene, a P450 hydroxylase gene, an ADP-glucose pyrophosphorylase gene, a proline dehydrogenase gene, an endo-1,4-beta-glucanase gene, a zeaxanthin epoxidase gene, and a 1-aminocyclopropane-1-carboxylate synthase gene.

86. A vector according to claim 65, further comprising a spacer element that is either an Ubi intron sequence or a GBSS spacer sequence.

87. A vector according to claim 65, wherein the terminator is a Ubi3 terminator sequence or a 3'-untranslated region of an endogenous plant gene.

88. A vector according to claim 65, further comprising a selectable marker gene operably linked to a constitutive promoter and a Cre gene operably linked to an inducible promoter, wherein the selectable marker gene and the Cre gene are flanked by a first recombinase recognition site and a second recombinase recognition site.

89. A vector according to claim 88, wherein the first recombinase recognition site and the second recombinase recognition site are lox sites.

90. A vector according to claim 88, wherein the inducible promoter is a temperature-sensitive promoter, a chemically-induced promoter, or a temporal promoter.

91. A vector according to claim 88, wherein the inducible promoter is a Ha hsp17.7 G4 promoter, a wheat wcs120 promoter, a Rab 16A gene promoter, an α-amylase gene promoter, a pin2 gene promoter, a carboxylase promoter.

92. A vector according to claim 65, further comprising a plant-derived marker gene.

93. A vector according to claim 92, wherein the plant-derived marker gene is an enolpyruvul-3-phosphoshikimic acid synthase gene, a salt-tolerance gene, or the PST1 gene, the PST2 gene, or the PST3 gene.

94. A method for modifying a plant cell, comprising integrating a P-DNA sequence into the genome of a plant cell, wherein the P-DNA consists essentially of, in the 5'- to 3'- direction, a first T-DNA border-like sequence, a promoter, a desired polynucleotide sequence operably linked to the promoter, a terminator, and a second T-DNA border-like sequence, wherein the border-like sequences have less than 100% sequence identity with T-DNA border sequences, and wherein the T-DNA border-like sequences, the promoter, the desired polynucleotide, and terminator, are all isolated from or native to the genome of the plant cell, wherein the desired polynucleotide comprises sense and antisense seqeunces of a leader sequence or trailer sequence that are associated with the upstream or downstream non-coding regions of a gene in the plant, and wherein expression of the desired polynucleotide produces a double-stranded RNA transcript that targets the gene associated with the desired polynucleotide, thereby modifying the plant cell.

95. A method for modifying a plant, comprising:

(i) transfecting at least one cell in the plant with the vector of claim 88;

(ii) selecting a cell expressing the selectable marker;

(iii) isolating the cell expressing the selectable marker;

(iii) inducing the expression of the Cre gene in the isolated cell;

(iv) culturing the isolated cell; and

(ii) observing the phenotype of cultured cells;

wherein a phenotype that is different to an untransfected plant cell indicates that the target plant cell has been modified.

96. A method according to claim 14 or 95, wherein the selecting step is performed by identifying which cells are resistant to an antibiotic.

97. A method for identifying a target plant cell whose genome contains a P-DNA, comprising co-transfecting a plant target cell with the vector of claim 65 and a second *Agrobacterium*-derived vector that comprises a marker gene flanked by a T-DNA borders or T-DNA border-like sequences and a omega-mutated virD2 gene, wherein the P-DNA of the vector of claim 65 is integrated into the genome of the plant target cell, and wherein no part of the second *Agrobacterium*-derived vector is integrated into the genome of the plant target cell, and wherein the omega-mutated virD2 gene is in the backbone.

98. A method according to claim 97, wherein the marker in the second *Agrobacterium*-derived vector is a neomycin phosphotransferase gene.

99. A method for identifying a target plant cell whose genome contains at least a part of an integration cassette according to claim 98, further comprising selecting cells that survive temporary growth on a kanamycin-containing media, wherein the genomes of the selected cells contain only the integration cassette.

100. A method according to any one of claims 94, 95, or 97, wherein the target plant cell is within a plant.

101. A plant comprising at least one cell whose genome comprises a P-DNA according to claim 65.

102. A plant comprising at least one cell whose genome is artificially manipulated to contain only plant-derived nucleic acids, wherein no cells of the plant contain foreign nucleic acids integrated into the cell genome.

103. The plant of claim 102, wherein the cell is capable of expressing at least one of the plant-derived nucleic acids, which expression modifies a trait associated with the plant.

104. A method for reducing black spot bruising in a selected plant species, comprising (i) integrating into a genome of a selected plant species, a P-DNA, delineated by border-like sequences, comprised only of polynucleotides native to, or isolated from the selected plant species, or comprised of polynucleotides native to, or isolated from a plant species that is sexually compatible with the selected plant species, wherein the P-DNA consists essentially of, in the 5'- to 3'- direction, a promoter; a sense-orientated leader nucleotide sequence from a PPO gene; an antisense-oriented sequence of the leader nucleotide sequence; and a termination sequence, wherein the promoter produces a double-stranded RNA molecule, and wherein the double-stranded RNA molecule brings about a reduction in the expression of the endogenous PPO gene, thereby reducing black spot bruising in the plant.

105. A method for reducing black spot bruising in a selected plant species, comprising integrating into a genome of a selected plant species a P-DNA, delineated by border-like sequences, comprised only of nucleotide sequences native to, or isolated from the selected plant species or comprised of polynucleotides native to, or isolated from a plant species that is sexually compatible with the selected plant species, wherein the P-DNA consists essentially of, in the 5'- to 3'- direction, a promoter; a sense-orientated trailer nucleotide sequence from a PPO gene; an antisense-oriented sequence of the trailer nucleotide sequence; and a termination sequence, wherein the promoter produces a double-stranded RNA molecule, and wherein the double-stranded RNA molecule brings about a reduction in the expression of the PPO gene, thereby reducing black spot bruising in the plant.

106. A method for reducing cold-induced sweetening in a selected plant species, comprising integrating into a genome of a selected plant species a P-DNA, delineated by border-like sequences, comprised only of nucleotide sequences isolated from the selected plant species or or comprised of polynucleotides native to, or isolated from a plant species that is sexually compatible with the selected plant species, wherein the P-DNA consists essentially of, in the 5'- to 3'- direction, a promoter; a sense-orientated leader nucleotide sequence associated with an R1 gene; an antisense-oriented sequence of the leader nucleotide sequence; and a termination sequence, wherein the promoter produces a double-stranded RNA molecule, and wherein the double-stranded RNA molecule reduces the expression of the R1 gene, thereby reducing cold-induced sweetening in the plant.

107. A method for reducing cold-induced sweetening in a selected plant species, comprising integrating into a genome of a selected plant species an P-DNA, delineated by border-like sequences, comprised only of nucleotide sequences isolated from the selected plant species or comprised of polynucleotides native to, or isolated from a plant species that is sexually compatible with the selected plant species, wherein the P-DNA consists essentially of, in the 5'- to 3'- direction, a promoter; a sense-orientated trailer nucleotide sequence associated with an R1 gene; an antisense-oriented sequence of the trailer nucleotide sequence; and a termination sequence, wherein the promoter produces a double-stranded RNA molecule, and wherein the double-stranded RNA molecule reduces the expression of the R1 gene, thereby reducing cold-induced sweetening in the plant.

108. The method of claim 8, wherein the sequence of interest is a gene.

109. The method of claim 108, wherein the gene is a modified polyphenol oxidase, polyphenol oxidase gene, or a modified R1 gene, or an R1 gene.

110. The method of claim 8, wherein the promoter is an inducible promoter.

111. The method of claim 8, wherein the terminator is a yeast ADH terminator sequence.

112. The method of claim 8, wherein the sequence of interest is a leader or trailer sequence, wherein the leader or trailer sequence represents a sequence upstream or downstream of a gene that is native to the plant cell.

113. The method of claim 112, wherein the sequence of interest comprises a sense-oriented leader sequence operably linked to an antisense leader sequence.

114. The method of claim 112, wherein the sequence of interest comprises a sense-oriented trailer sequence operably linked to an antisense trailer sequence.

115. The method of claim 113, wherein the leader construct comprises in 5'-to 3'-direction, a promoter, a sense-oriented leader sequence, the antisense sequence of the leader, and a terminator, wherein expression of the leader construct produces a double-stranded RNA molecule that facilitates the down-regulation of expression of the gene to which it is associated.

116. The method of claim 115, wherein the leader sequence is associated with, and located upstream of, the coding region of the PPO gene, the R1 gene, an L-type phosphorylase gene, or an alpha glucan phosphorylase gene.

117. The method of claim 113, wherein the trailer construct comprises in 5'-to 3'-direction, a promoter, a sense-oriented trailer sequence, the antisense sequence of the trailer, and a terminator, wherein expression of the trailer construct produces a double-stranded RNA molecule that facilitates the down-regulation of expression of the gene to which it is associated.

118. The method of claim 117, wherein the trailer sequence is associated with, and located downstream of, the coding region of the PPO gene, the R1 gene, an L-type phosphorylase gene, or an alpha glucan phosphorylase gene.

119. The method of claim 9, further comprising exposing the plant cell to a second vector that comprises a marker element, wherein the marker is transiently expressed in the transformed plant and is not stably integrated into the genome of the transformed plant.

120. The method of claim 119, wherein the marker is a herbicide resistance gene.

121. The method of claim 120, wherein the cytokinin gene is an antibiotic resistance gene.

122. The method of claim 119, wherein the marker is a NPTII.

123. A polynucleotide comprising the polynucleotide sequence of SEQ ID NO. 93, wherein the polynucleotide is between 20 and 80 nucleotides in length.

124. The polynucleotide of claim 123, wherein the polynucleotide is between 21 and 70 nucleotides in length.

125. The polynucleotide of claim 123, wherein the polynucleotide is between 22 and 50 nucleotides in length.

126. The polynucleotide of claim 123, wherein the polynucleotide is between 23 and 40 nucleotides in length.

127. The polynucleotide of claim 123, wherein the polynucleotide is between 24 and 30 nucleotides in length.

128. The method of claim 4, wherein the plant cells are transfected with the first polynucleotide before the second polynucleotide.

129. A tuber-specific promoter as shown in SEQ ID NO. 40.

130. An *Agrobacterium*-based method of making transgenic plant cells that do not contain a selectable marker gene stably integrated in nuclear DNA comprising:

a. constructing a first binary vector comprised of a polynucleotide consisting essentially of a desired gene operably linked to T-DNA borders or T-DNA border-like sequences at the 5' and 3' ends of said desired gene;

b. constructing a second binary vector comprised of a selectable marker gene operably linked to T-DNA borders or T-DNA border-like sequences at the 5' and 3' ends of said selectable marker gene;

c. incubating plants cells with:

i. an *Agrobacterium* strain carrying said first and said second binary vectors; or

ii. a first *Agrobacterium* strain carrying said first binary vector and a second *Agrobacterium* strain carrying said second binary vector;

d. selecting plant cells wherein said desired gene is integrated into plant nuclear DNA without integration of said selectable marker gene into plant nuclear DNA following incubation for an appropriate time period on a medium containing an appropriate selection agent.

131. The method according to claim 130, wherein said selectable marker gene is a herbicide resistance gene or an antibiotic resistance gene.

132. The method according to claim 131, wherein said antibiotic resistance gene is the nptII gene.

133. The method according to claim 132, wherein said antibiotic resistance gene is the npt II structural gene operably linked to the promoter from the Ubiquitin-7 gene and the termninator from yeast alcohol dehydrogenase 1 (ADH1) gene.

134. The method according to claim 130, wherein said plant cells are first incubated with said first *Agrobacterium* strain and then subsequently incubated with said second *Agrobacterium* strain or vice versa.

135. The method according to claim 130, wherein said first binary vector further comprises a binary integration marker gene that can be used to detect plant cells stably transformed with binary vector backbone sequences.

136. The method according to claim 135, wherein said binary vector integration marker gene is selected from the group consisting of a herbicide resistance gene, antibiotic resistance gene, or NPTII.

137. The method according to claim 130, wherein said second binary vector further comprises a gene fusion between the bacterial cytosine deaminase (*codA*) and uracil phophoribsyltransferase (*upp*) genes, which is inserted between the T-DNA or T-DNA border-like sequences, and plant cells are exposed to 5-fluorocytosine following incubation with said first and second *Agrobacterium* strains in order to select against those plant cells transformed with said second binary vector.

138. The method according to claim 130, wherein said secondary binary vector further comprises the omega-mutated *virD2* gene, wherein said omega-mutated *virD2* gene reduces the frequency of integration of said selectable marker gene into said plant nuclear DNA.

139. The isolated, border-like nucleotide sequence of claim 44, wherein the sequence is 25 nucleotides in length.

140. The method of claim 61, wherein the second binary vector comprises at least one of a negative selectable marker gene and a gene that impairs integration, wherein the negative selectable marker gene is positioned within the right T-DNA border and the left T-DNA border, and wherein the gene that impairs integration is positioned within the backbone of the second binary vector.

141. The method of claim 99, wherein the temporary growth is from 1 to 5 days.

Figure 1. Diagrams for some P-DNA vectors

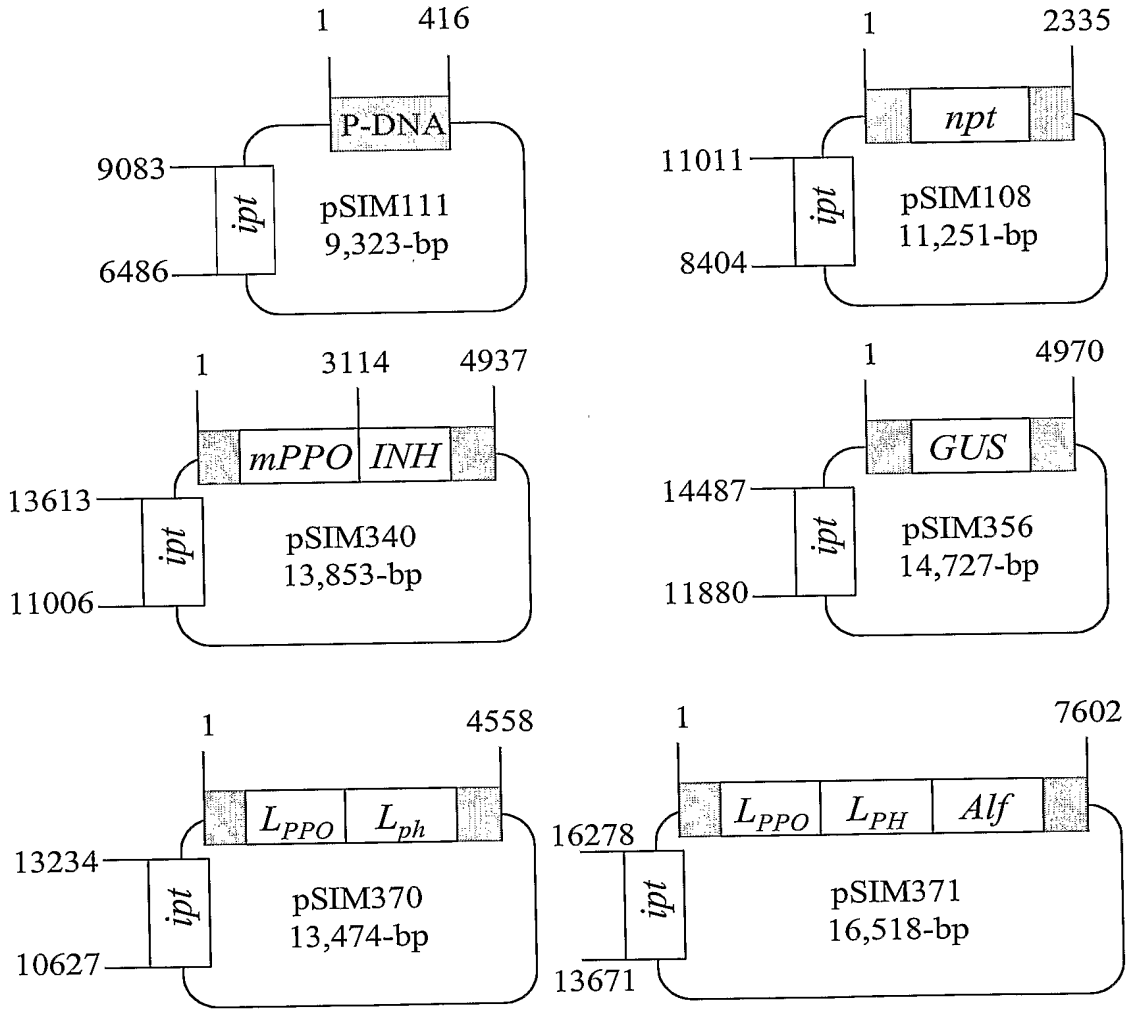


Figure 2. Alignment of potato and tobacco invertase inhibitor proteins

A.

```
St-inh1         MRNLFPILMLITNLALNNDNNNNNNNNNNYNLIHATCRETPYYSLCLTTLQSGPRSNEVE 60
Nt-inhh         MRNLFPIFMLITNLAFN-DNNNSNN------IINTTCRATTNYPLCLTTLHSDPRTSEAE 53
                *******:*******:* ****.**      :*::*** *. *.******:*.**:.*.*

St-inh1         GGDAITTLGLIMVDAVKSKSIEIMEKIKELEKSNPEWRAPLSQCYVAYNAVLRADVTVAV 120
Nt-inhh         GAD-LTTLGLVMVDAVKLKSIEIMKSIKKLEKSNPELRLPLSQCYIVYYAVLHADVTVAV 112
                *.* :*****:****** ******:.**:******* * ******:.* ***:*******

St-inh1         EALKKGAPKFAEDGMDDVVAEAQTCEYSFNYYNKLDFPISNLSREIIELSKVAKSIIRML 180
Nt-inhh         EALKRGVPKFAENGMVDVAVEAETCEFSFK-YNGLVSPVSDMNKEIIELSSVAKSIIRML 171
                ****:*.*****:** **..**:***:**: ** *  *:*::.:******.*********

St-inh1         L 181
Nt-inhh         L 172
                *
```

B.

```
St-inh1         MRNLFPILMLITNLALNNDNNNNNNNNNNYNLIHATCRETPYYSLCLTTLQSGPRSNEVE 60
Nt-inh1         MKNLIFLTMFLTILLQTNANN----------LVETTCKNTPNYQLCLKTLLSDKRS--AT 48
                *:**: : *::* *  .* **          *:.:**::** *.***.** *. **  .

St-inh1         GGDAITTLGLIMVDAVKSKSIEIMEKIKELEKSNP--EWRAPLSQCYVAYNAVLRADVTV 118
Nt-inh1         G--DITTLALIMVDAIKAKANQAAVTISKLRHSNPPAAWKGPLKNCAFSYKVILTASLPE 106
                *   ****.******:*:*: :   .*.:*.:***   *:.**.:* .:*:.:* *.:.

St-inh1         AVEALKKGAPKFAEDGMDDVVAEAQTCEYSFNYYNKLDFPISNLSREIIELSKVAKSIIR 178
Nt-inh1         AIEALTKGDPKFAEDGMVGSSGDAQECE---EYFKGSKSPFSALNIAVHELSDVGRAIVR 163
                *:***.** ******** .  .:** **   :*::  . *:* *.  : ***.*.::*:*

St-inh1         MLL 181
Nt-inh1         NLL 166
                **
```

Figure 3. Gene-free expression cassettes

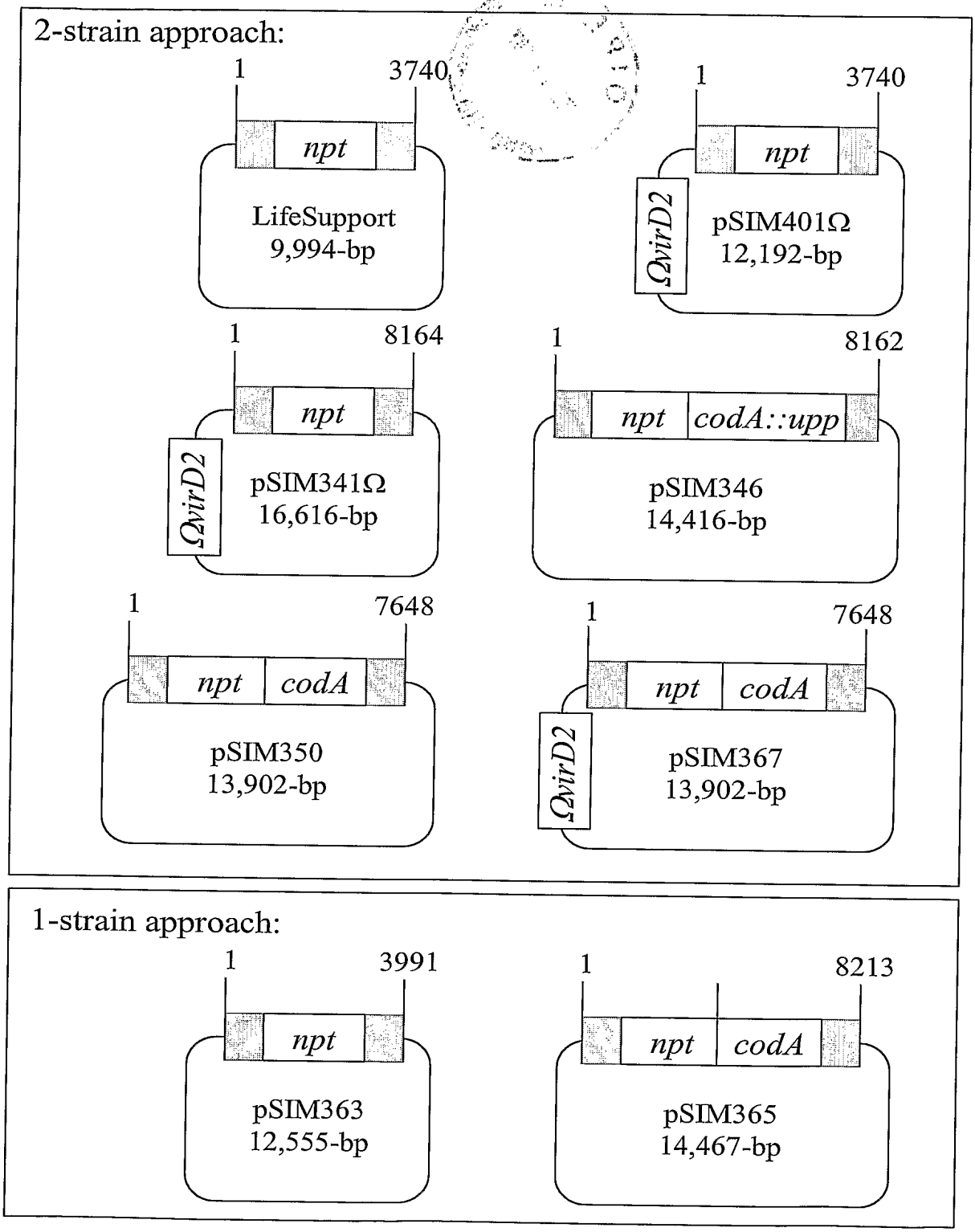
Promoter	*SOI**	*Spacer*	*Total size*
P:GBSS-small	Leader a/w** *R1*	Ubi intron	1729-bp
P:GBSS-small	Leader a/w*R1*	GBSS spacer	1397-bp
P:GBSS-large	Leader a/w*R1*	Ubi intron	2005-bp
P:GBSS-large	Leader a/w*R1*	GBSS spacer	1397-bp
P:GBSS-small	Trailer a/w*R1*	GBSS spacer	2042-bp
P:GBSS-small	Trailer a/w*R1*	Ubi intron	1705-bp
P:GBSS-large	Trailer a/w*R1*	GBSS spacer	2313-bp
P:GBSS-large	Trailer a/w*R1*	Ubi intron	1981-bp
P:GBSS-small	Leader a/w*Phosph.*	GBSS spacer	-
P:GBSS-small	Leader a/w*Phosph.*	Ubi intron	-
P:GBSS-large	Leader a/w*Phosph.*	GBSS spacer	1852-bp
P:GBSS-large	Leader a/w*Phosph.*	Ubi intron	2184-bp
P:GBSS-large	Leader a/w*PPO*	Ubi intron	1958-bp
P:GBSS-large	Leader a/w*PPO*	GBSS spacer	1626-bp
P:GBSS-small	Leader a/w*PPO*	Ubi intron	-
P:GBSS-small	Leader a/w*PPO*	GBSS spacer	-

*: sequence-of-interest; **: "associated with"

Figure 4. Alignment of the 3'-end of tuber-expressed PPO genes and trailers associated with these genes. Stop codons ('TAA') are underlined. The trailer sequence used for genetic modification of potato plants was isolated from 'P-PPO3', and is downstream from the stop codon (TTAGTC...ACAATT).

```
P-PPO3          CTGGCGATAACGGAACTGTTGGAGGATATTGGTTTGGAAGATGAAGATACTATTGCGGTG 60
PPOM-41         CTGGCGATAACGGAACTGTTGGAGGATATTGGATTGGAAGATGAAGATACTATTGCGGTA 60
PPOM-44         CTGGCGATAACGGAACTGTTGGAGGATAATGGATTGGAAGATGAAGGTACTATNGCGGTA 60

P-PPO3          ACTCTGGTGCCAAAGAGAGGTGGTGAAGGTATCTCCATTGAAAGTGCGACGATCAGTCTT 120
PPOM-41         ACTTTGGTTCCAAAAGTAGGTGGTGAAGGTGTATCCATTGAAAGTGTGGAGATCAAGCTT 120
PPOM-44         ACTTTGGTTCCAAAAGTTGGTGGTGAAGGTGTATCCATTGAAAGTGCGGAGATCAAGCTT 120

P-PPO3          GCAGATTGTTAATTAGTCTCTA-TTGA-ATCTGCTG----AGATTACAC-TTTGATGGAT 173
PPOM-41         GAGGATTGTTAAGTCCTCATGAGTTGGTGGCTACGGTACCAAATTTTATGTTTAATTAGT 180
PPOM-44         GAGGATTGTTAAGTCCTCATGAGTTGGTGGCTATGGTACCAAATTNTATGTTTAATTAGT 180

P-PPO3          GATGCTCTGTT--TTTGTTTTCTTGTTCTGTTTTTTCCTC-TGTTGAAATCAGCTTTGTT 230
PPOM-41         ATTAATGTGTGTATGTGTTTGATTATGTTTCGGTTAAAATGTATCAGCTGGATAGCTGAT 240
PPOM-44         ATTAATGTGTG----TGTTTGATTATGTTTCGGTTAAAATGTATCANCTGGATAGCTGAT 236

P-PPO3          -GCTTGATTTC---ATTGAAGTTGTTATTCAAGAA-TAAATCAGTTA-CAATT------- 277
PPOM-41         TACTAGCCTTGCCAGTTGTTAATGCTATGTATGAAATAAATAAATAAATGGTTGTCTTCT 300
PPOM-44         TACTAGCCTTCCCAGTTGTTAATGCTATGTATGAAATACATAAATAAATGGTTGTCTTCC 296
```

Figure 5. Diagrams for some LifeSupport vectors



SEQ ID NOs.

SEQ ID NO.: 1:	Potato P-DNA. The bold underlined portions of SEQ ID NO. 1 represent the left (5'-) and right (3'-) border-like sequences of the P-DNA respectively.
SEQ ID NO.: 2:	Wheat P-DNA
SEQ ID NO.: 3:	Expression cassette for the IPT gene
SEQ ID NO.: 4:	Binary vectors pSIM111
SEQ ID NO.: 5:	Potato invertase inhibitor gene
SEQ ID NO.: 6:	Potato GBSS promoter
SEQ ID NO.: 7:	Potato Ubiquitin-3 gene terminator
SEQ ID NO.: 8:	Potato leader associated with the *R1* gene
SEQ ID NO.: 9:	Potato Ubiquitin intron
SEQ ID NO.: 10:	Expression cassette for a sense and antisense copy of the leader associated with the *R1* gene
SEQ ID NO.: 11:	Spacer
SEQ ID NO.: 12:	Alternative expression cassette for a sense and antisense copy of the leader associated with the *R1* gene
SEQ ID NO.: 13:	Longer potato GBSS promoter
SEQ ID NO.: 14:	Alternative expression cassette for a sense and antisense copy of the leader associated with the *R1* gene
SEQ ID NO.: 15:	Alternative expression cassette for a sense and antisense copy of the leader associated with the *R1* gene
SEQ ID NO.: 16:	Potato trailer associated with the *R1* gene
SEQ ID NO.: 17:	Expression cassette for a sense and antisense copy of the trailer associated with the *R1* gene
SEQ ID NO.: 18:	Expression cassette for a sense and antisense copy of the trailer associated with the *R1* gene
SEQ ID NO.: 19:	Expression cassette for a sense and antisense copy of the trailer associated with the *R1* gene
SEQ ID NO.: 20:	Expression cassette for a sense and antisense copy of the trailer associated with the *R1* gene
SEQ ID NO.: 21:	Potato leader associated with the L glucan phosphorylase gene
SEQ ID NO.: 22:	Potato trailer associated with the L glucan phosphorylase gene
SEQ ID NO.: 23:	Expression cassette for a sense and antisense copy of the leader associated with the L glucan phosphorylase gene
SEQ ID NO.: 24:	Alternative expression cassette for a sense and antisense copy of the leader associated with the L glucan phosphorylase gene
SEQ ID NO.: 25:	Alternative expression cassette for a sense and antisense copy of the leader associated with the L glucan phosphorylase gene
SEQ ID NO.: 26:	Alternative expression cassette for a sense and antisense copy of the leader associated with the L glucan phosphorylase gene
SEQ ID NO.: 27:	Potato *PPO* gene
SEQ ID NO.: 28:	Modified inactive potato *PPO* gene
SEQ ID NO.: 29:	Potato trailer associated with a *PPO* gene
SEQ ID NO.: 30:	Expression cassette for a sense and antisense copy of the trailer associated with a *PPO* gene

SEQ ID NO.: 31: Alternative expression cassette for a sense and antisense copy of the trailer associated with a *PPO* gene

SEQ ID NO.: 32: Alternative expression cassette for a sense and antisense copy of the trailer associated with a *PPO* gene

SEQ ID NO.: 33: Alternative expression cassette for a sense and antisense copy of the trailer associated with a *PPO* gene

SEQ ID NO.: 34: Potato trailer associated with a starch branching enzyme gene

SEQ ID NO.: 35: Potato trailer associated with a starch branching enzyme gene

SEQ ID NO.: 36: Expression cassette for an omega-mutated *virD2* gene

SEQ ID NO.: 37: Potato salt tolerance gene *Pst1*

SEQ ID NO.: 38: Potato salt tolerance gene *Pst2*

SEQ ID NO.: 39: Potato salt tolerance gene *Pst3*

SEQ ID NO.: 40: Potato tuber specific promoter

SEQ ID NO.: 56: Yeast ADH terminator

SEQ ID NO. 94: Wheat left border-like sequence

SEQ ID NO. 95: Wheat right border-like sequence

SEQID1

GTTTACAGTACCATATATCCTGTCAGAGGTATAGAGGCATGACTGGCATGATCACTAAATTGATGCCCACAGAGGAGACTTATAAC
CTACAGGGGCACGTAGTTCTAGGACTTGAAAGTGACTGACCGTAGTCCAACTCGGTATAAAGCCTACTCCCAACTAAATATATGAA
ATTTATAGCATAACTGCAGATGAGCTCGATTCTAGAGTAGGTACCGAGCTCGAATTCCTTACTCCTCCACAAAGCCGTAACTGAAG
CGACTTCTATTTTTCTCAACCTTCGGACCTGACGATCAAGAATCTCAATAGGTAGTTCTTCATAAGTGAGACTATCCTTCATAGCT
ACACTTTCTAAAGGTACGATAGATTTTGGATCAACCACACACACTTCGTTTACACCGGTATATATCCTGCCA

SEQID2

TGGCAGGATATATGAGTGTGTAAACAACCATAATCAGGCTGTAATTATCAAGAGAACTAATGACAAGAAGCAGAGCTTATCAAGTG
TTTCGTCCAGCTGTAACATGGGCACAAAAGCTTGCTTGATGCATGTCTGGCTTTTCAAAGAGCAATGTATTCTCAGGTACCTGCAC
GTTTGATCCCCTACCACGTACAAGACGAGCAGAAAGGACATGTCTGCAGAAACTTAGACACATCCATTGCAGACTCGTTCCGAAGC
ATCAGGAGAGTAGTCAGCAATGGTCATCTGCTGATGTAAATTAATTGATTGTTGGTAATCAAATTTTAACAGCAATATATATAATA
TATCAATAGTATATTGAACTATGAAAGACTGTAATCATATATAACAGCATACAAATTGTCGTGGAAACAAGAGGAGCTCATCAAGT
GTTTAGTTCAGAAATAGCTAACCAAGAATGCAATATAATAGGGGTACTGAGCTCCCTTCAAAATTACTAACTTCAGAAATAGCTAA
CCAAGAATGCAATGGCATTGCATAATTTAAACAACTGTCAGCACCAATCTCTGACTGAAGGCAGTTTACCCATTCAGAAGAGCACA
CATTTTCTGAACGACAACTCTGAGCGGGGATTGTTGACAGCAGCAATTAATCTGGCCTCAAGATGGTTTCCAACAACATAGATCAG
ATACAGCACTCAAGCACCCAATAATCAGCCAGTACTGATCTGGTTACCACTGCAATTGATTAACAGATGAACTGTGAAATTAAGAT
TTAACTGACAGTAATATATACCAGTTGGCAGGATATATCCCTCTGTAAAC

SEQID3

CTGCAGCCAAAGCACATACTTATCGATTTAAATTTCATCGAAGAGATTAATATCGAATAATCATATACATACTTTAAATACATAAC
AAATTTTAAATACATATATCTGGTATATAATTAATTTTTTAAAGTCATGAAGTATGTATCAAATACACATATGGAAAAAATTAACT
ATTCATAATTTAAAAAATAGAAAAGATACATCTAGTGAAATTAGGTGCATGTATCAAATACATTAGGAAAAGGGCATATATCTTGA
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AACATTCAACAAAGTAACAACCAACTAGAGTGAAAAATACACATTGTTCTCTAAACATACAAAATTGAGAAAAGAATCTCAAAATT
TAGAGAAACAAATCTGAATTTCTAGAAGAAAAAAATAATTATGCACTTTGCTATTGCTCGAAAAATAAATGAAAGAAATTAGACTT
TTTTAAAAGATGTTAGACTAGATATACTCAAAAGCTATCAAAGGAGTAATATTCTTCTTACATTAAGTATTTTAGTTACAGTCCTG
TAATTAAAGACACATTTTAGATTGTATCTAAACTTAAATGTATCTAGAATACATATATTTGAATGCATCATATACATGTATCCGAC
ACACCAATTCTCATAAAAAGCGTAATATCCTAAACTAATTTATCCTTCAAGTCAACTTAAGCCCAATATACATTTTCATCTCTAAA
GGCCCAAGTGGCACAAAATGTCAGGCCCAATTACGAAGAAAAGGGCTTGTAAAACCCTAATAAAGTGGCACTGGCAGAGCTTACAC
TCTCATTCCATCAACAAAGAAACCCTAAAAGCCGCAGCGCCACTGATTTCTCTCCTCCAGGCGAAGATGCAGATCTTCGTGAAGAC
CCTAACGGGGAAGACGATCACCCTAGAGGTTGAGTCTTCCGACACCATCGACAATGTCAAAGCCAAGATCCAGGACAAGGAAGGGA
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GAAGCGGACGACCAACAGTGGAAGAACTGAAAGGAACGACGCGTCTCTACCTTGATGATCGGCCTCTGGTGGAGGGTATCATCGCA
GCCAAGCAAGCTCATCATAGGCTGATCGAGGAGGTGTATAATCATGAGGCCAACGGCGGGCTTATTCTTGAGGGAGGATCCACCTC
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CCTTCATGAAAGCGGCCAAGGCCAGAGTTAAGCAGATGTTGCACCCCGCTGCAGGCCATTCTATTATTCAAGAGTTGGTTTATCTT
TGGAATGAACCTCGGCTGAGGCCCATTCTGAAAGAGATCGATGGATATCGATATGCCATGTTGTTTGCTAGCCAGAACCAGATCAC
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GCCCTGCGTCGCACCTGTCTTCATCTGGATAAGATGTTCGTAATTGTTTTTGGCTTTGTCCTGTTGTGGCAGGGCGGCAAATACTT
CCGACAATCCATCGTGTCTTCAAACTTTATGCTGGTGAACAAGTCTTAGTTTCCACGAAAGTATTATGTTAAATTTTAAAATTTCG
ATGTATAATGTGGCTATAATTGTAAAAATAAACTATCGTAAGTGTGCGTGTTATGTATAATTTGTCTAAATGTTTAATATATATCA

TAGAACGCAATAAATATTAAATATAGCGCTTTTATGAAATATAAATACATCATTACAAGTTGTTTATATTTCGGGTGGACTAGTTT
TTAATGTTTAGCAAATGTCCTATCAGTTTTCTCTTTTTGTCGAACGGTAATTTAGAGTTTTTTTTGCTATATGGATTTTCGTTTTT
GATGTATGTGACAACCCTCGGGATTGTTGATTTATTTCAAAACTAAGAGTTTTTGCTTATTGTTCTCGTCTATTTTGGATATCAAT
CTTAGTTTTATATCTTTTCTAGTTCTCTACGTGTTAAATGTTCAACACACTAGCAATTTGGCTGCAGCGTATGGATTATGGAACTA
TCAAGTCTGTGGGATCGATAAATATGCTTCTCAGGAATTTGAGATTTTACAGTCTTTATGCTCATTGGGTTGAGTATAATATAGTA
AAAAAATAGGAATTC

SEQID4
AGCTTTGGCAGGATATATACCGGTGTAAACGAAGTGTGTGTGGTTGATCCAAAATCTATCGTACCTTTAGAAAGTGTAGCTATGAA
GGATAGTCTCACTTATGAAGAACTACCTATTGAGATTCTTGATCGTCAGGTCCGAAGGTTGAGAAAAAATAGAAGTCGCTTCAGTTA
CGGCTTTGTGGAGGAGTAAGGGTACCTACTCTAGAATCGAGCTCATCGTTATGCTATAAATTTCATATATTTAGTTGGGAGTAGGC
TTTATACCGAGTTGGACTACGGTCAGTCACTTTCAAGTCCTAGAACTACGTGCCCCTGTAGGTTATAAGTCTCCTCTGTGGGCATC
AATTTAGTGATCATGCCAGTCATGCCTCTATACCTCTGACAGGATATATGGTACTGTAAACACTAGTTGTGAATAAGTCGCTGTGT
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TATAGTGCAGTCGGCTTCTGACGTTCAGTGCAGCCGTCTTCTGAAAACGACATGTCGCACAAGTCCTAAGTTACGCGACAGGCTGC
CGCCCTGCCCTTTTCCTGGCGTTTTCTTGTCGCGTGTTTTAGTCGCATAAAGTAGAATACTTGCGACTAGAACCGGAGACATTACG
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SEQID6
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SEQID16
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SEQID17
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SEQID18
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SEQID19
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SEQID20
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SEQID21
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SEQID22
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SEQID23
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SEQID24
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SEQID25
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SEQID26

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SEQID27

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SEQID28

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SEQID29
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SEQID 30
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SEQID31
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SEQID32
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SEQID33
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SEQID34
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SEQID35
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SEQID36
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SEQID37
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SEQID38
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SEQID39
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SEQID40
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SEQID56
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SEQID 95
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